(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 142 481 A1

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 158(3) EPC

(43) Date of publication:
10.10.2001 Bulletin 2001/41

(21) Application number: **00900164.5**

(22) Date of filing: **11.01.2000**

(51) Int Cl.7: **A23C 9/123**, A23C 20/02

(86) International application number:
PCT/JP00/00068

(87) International publication number:
WO 00/41572 (20.07.2000 Gazette 2000/29)

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **11.01.1999 JP 394699**

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(54) PROCESS FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND PROCESS FOR PRODUCING MILK SERUM

(57) There are disclosed methods for producing fermented milk and whey that enable effective production in high yield of fermented milk and whey having high content of an ACEi peptide that is highly safe and applicable to pharmaceuticals, functional foods, health foods, and the like. The methods are: a method including the steps of mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material, and fermenting the mixed material under stirring so that curd pieces and whey containing an angiotensin converting enzyme inhibitory peptide are generated, whereby fermented milk containing the curd pieces and the whey containing the angiotensin converting enzyme inhibitory peptide is produced; and a method including the steps of subjecting the resulting fermented milk to centrifugation and/or filter pressing to separate and recover whey.

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Printed by Jouve, 75001 PARIS (FR)

Description

[0001] The present invention relates to a method for producing fermented milk containing an angiotensin converting enzyme inhibitory peptide which enables effective production of fermented milk containing an angiotensin converting enzyme inhibitory peptide such as Val-Pro-Pro and/or Ile-Pro-Pro, and to a method for producing whey containing an angiotensin converting enzyme inhibitory peptide which enables effective separation and production of whey containing an angiotensin converting enzyme inhibitory peptide.

[0002] Angiotensin Converting Enzyme (abbreviated as "ACE" hereinbelow) is found mainly in lungs or vascular endothelial cells. ACE acts on angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Pro-Phe-His-Leu), which has been generated by digestion with renin, to release a dipeptide (His-Leu) from its C-terminal, thereby giving angiotensin II, which oauses contraction of vascular smooth muscle and has strong hypertensive effect. This enzyme also decomposes and inactivates bradykinin, which has hypotensive effect. Such ACE produces hypertensive peptide (angiotensin II) and at the same time inactivates hypotensive peptide (bradykinin), so that it exhibits hypertensive effect. Therefore, angiotensin converting enzyme inhibitor (abbreviated as ACEI hereinbelow), which inhibits activity of ACE, has hypertension inhibitory effect.

[0003] As ACEI, there are known peptides having three to ten amino acid resldues including Val-Pro-Pro (Japanese Patent No. 2782142) and a tripeptide Ile-Pro-Pro (JP-A-3-120225). There is also known a peptide having ACEI activity, which is produced by digestion of milk casein by protease produced by lactic acid bacteria, and found in dissolved state in whey of fermented milk (J. Dairy Sci. 78, 6, p1253-1257, 1995).

[0004] Such peptides as ACEI may be taken in the form of fermented milk per se containing Val-Pro-Pro and/or Ile-Pro-Pro. However, in view of the concentration and effective dose of the peptides as ACEI in the fermented milk, it is necessary to take a considerable amount of fermented milk. Thus, development of a method for producing fermented milk or whey containing a large amount of ACEI has been demanded.

[0005] It is known that ACEI such as Val-Pro-Pro and/or Ile-Pro-Pro is highly safe and thus can be used for pharmaceuticals, functional foods, health foods, and the like. For producing Val-Pro-Pro and/or Ile-Pro-Pro, there is proposed a method including the steps of culturing lactic acid bacteria in a medium containing peptides and/or proteins that have Val-Pro-Pro and/or Ile-Pro-Pro units to prepare fermented milk, and purifying the fermented milk (Japanese Patent No. 2782153).

[0006] Conventional lactic acid fermentation, for example for production of typical fermented milk products such as yogurt, is carried out by mixing starter bacteria and a starting material by stirring to form a uniform mixture, and then fermenting the mixture under static conditions in order to make the resulting product as a whole in the form of a curd. Such static conditions are believed to be required because, when a fermentation liquid is at reduced pH due to fermentative proliferation of lactic acid bacteria, application of vibration, such as by stirring or shaking, to such fermentation liquid will cause whey off and coarse texture of the resulting fermented milk products. Further, the lactic acid bacteria for the lactic acid fermentation are facultative anaerobic, so that their growth is often inhibited by oxygen. Accordingly, it has never been intended at all to effect culturing under stirring during the period where the lactic acid fermentation under static conditions is required. In cheese production, too, the fermentation is carried out by mixing starter bacteria and a starting material by stirring to form a uniform mixture, fermenting the mixture under static conditions, and then coagulating casein by the action of rennet under static conditions, after which the reaction mixture is stirred and pressed for removing whey.

[0007] Improvement in whey recovery is required for industrial purification of whey from fermented milk followed by concentration of its active components. A variety of methods for recovering the curd fraction from fermented milk have hitherto been proposed, but effective separation of whey from fermented milk has hardly been performed to date.

[0008] It is an object of the present invention to provide methods for preparing fermented milk and whey containing an ACEI peptide which enable effective production in high yield of fermented milk and whey having high content of an ACEI peptide that is highly safe and applicable to pharmaceuticals, functional foods, health foods, and the like.

[0009] According to the present invention, there is provided a method for producing fermented milk containing an ACEI peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material, and
(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated,

whereby fermented milk containing said curd pieces and said whey containing the ACEI peptide is produced.

[0010] According to the present invention, there is also provided a method for producing fermented milk containing an ACEI peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material,

(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated, and
(B-2) fermenting said mixed material under static conditions,

whereby fermented milk containing said curd pieces and said whey containing the ACEI peptide is produced.

[0011] According to the present invention, there is further provided a method for producing whey containing an ACEI peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material,
(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated,

whereby fermented milk containing said curd pieces and said whey containing the ACEI peptide is produced, and subjecting the fermented milk to at least one of centrifugation and filter pressing to separate and recover whey.

[0012] According to the present invention, there is also provided a method for producing whey containing an ACEI peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material,
(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated,
(B-2) fermenting said mixed material under static conditions,

whereby fermented milk containing said curd pieces and said whey containing the ACEI peptide is produced, and subjecting the fermented milk to at least one of centrifugation and filter pressing to separate and recover whey.

[0013] The present invention will now be explained in detail.

[0014] The present methods include the step of mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material.

[0015] The milk as a starting material may be, for example, animal milk such as cow's milk, goat's milk, or sheep's milk; vegetable milk such as soy bean milk; or processed animal or vegetable milk such as skim milk, reconstituted milk, powdered milk, or condensed milk. These may be used as a mixture. Such milk contains peptides and proteins having Val-Pro-Pro and/or Ile-Pro-Pro units.

[0016] The solid content of the milk is not particularly limited. For example, when skim milk is used for production of fermented milk, the milk solid-non-fat content thereof is usually about 9 wt%. However, considering the productivity per facility, the milk solid-non-fat content is preferably raised to a certain degree in order to keep the production cost at a lower level. When the lactic acid fermentation under ordinary static conditions only is carried out at the milk solid-non-fat content of 13 wt% or higher, the viscosity of the resulting fermented milk becomes high, which will cause difficulties in separation of whey. Thus, the milk solid-non-fat content cannot be raised in the ordinary static fermentation. On the contrary, the methods of the present invention keep the resulting fermented milk at low viscosity even at the milk solid-non-fat content of 15 wt% or higher, since the fermentation in the present methods is accompanied by stirring as will be discussed later. Thus, whey can be obtained easily and efficiently.

[0017] In the methods of the present invention, the starting material may optionally contain other materials than milk as long as the object of the present invention is achieved. Such other materials may suitably be selected from the materials conventionally used in production of fermented milk, depending on the desired results.

[0018] The lactic acid bacteria used in the methods of the present invention are preferably those of the genus Lactobacillus. Examples of such lactic acid bacteria may include *Lactobacillus helveticus, Lactobacillus delbruekii* subsp. *bulgaricus, Lactobacillus acidophilus,* and the like. In particular, *Lactobacillus helveticus* CM4 (NATIONAL INSTITUTE OF BIOSCIENCE AND HUMAN TECHNOLOGY, AGENCY OF INDUSTRIAL SCIENCE AND TECHNOLOGY, Deposit No. FERM BP-6060, Deposition date: August 15, 1997) (referred to as *Lactobacillus helveticus* CM4 hereinbelow) is preferred as ACEI peptide-productive lactic acid bacteria. *Lactobacillus helveticus* CM4 under the deposit number mentioned above has been accepted for deposit under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. All restrictions on the availability to the public of FERM BP-6060 will be irrevocably removed upon the granting of a patent.

[0019] In the present invention, the lactic acid bacteria are preferably in the form of a precultured starter having sufficient activity. The initial cell count of the starter is preferably about 10^5 to 10^7 cells/ml.

[0020] In the present invention, other microorganisms may optionally be added to the mixed material as long as the object of the present invention is achieved. For example, yeast may additionally be used for improving the flavor and palatability of the resulting fermented milk or whey as functional food, health food, and the like.

[0021] Strains of the yeast are not particularly limited, and for example, yeast of the genus Saccharomyces such as

Saccharomyces cerevisiae may preferably be used. The content of the yeast may suitably be selected, depending on the desired result.

[0022] In the methods of the present invention, the mixing by stirring for preparing a mixed material may be conducted by a conventional method so that the lactic acid bacteria and the starting material are uniformly mixed. Incidentally, this mixing (A) is a conventional step, and distinguished from the fermentation step to be disoussed later.

[0023] The methods of the present invention include (B-1) fermenting the mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated, or this fermenting (B-1) and (B-2) fermenting said mixed material under static conditions, whereby fermented milk containing the curd pieces and the whey containing the ACEI peptide is produced.

[0024] These steps are for lactic acid fermentation of the mixed material. Conventional lactic acid fermentation has been effected under static conditions so that the mixed material as a whole turns to a lump such as a curd.

[0025] In the methods of the present invention, the conditions of the lactic acid fermentation and the final acidity for terminating the fermentation may suitably be set optimally, taking the amount of the ACEI peptide to be generated into account, since the optimum conditions vary depending on the species and strains of lactic acid bacteria, as well as on the milk solid content. For example, when *Lactobacillus helveticus* CM4 is used, the optimum temperature is 25 to 40 °C, and the duration of the fermentation is about 12 to 40 hours. The final acidity for terminating the fermentation is preferably about 1.5 to 3 wt % (weight percent of lactic acid).

[0026] In step (B-1), the fermentation is effected under stirring. When the lactic acid fermentation is effected only by step (B-1), the fermentation is effected under substantially continuous stirring. On the other hand, when the fermentation is effected by steps (B-1) and (B-2), each of the steps may be conducted at least once, and preferably conducted a plurality of times. In this case, the order of the steps is not particularly limited. The conditions for the stirring, and the conditions for the stirring and the standing may suitably be decided as long as a number of curd pieces and whey containing the ACEI peptide are generated in the fermentation step or steps. Preferably, such conditions may be decided so that the resulting mixture which contains the curd pieces and the whey containing the ACEI peptide has a viscosity of not higher than 20 cp, more preferably not higher than 10 cp. Here, the lower limit of the viscosity is not particularly imposed, but is usually about 2.0 cp. The generation of the curd pieces and whey can be achieved, for example, by setting the conditions so that the stirring is conducted while pH is lowered as the fermentation proceeds from about pH 5, at which soft curds are started to be generated, to pH 4.7-4.6, which is the isoelectric point of casein.

[0027] With the conventional fermentation only by stationary culture, curd is generated in the form of a plain-yogurt-like gel that is substantially contiguous all over the volume of a fermenter (tank). Such fermented milk curd cannot be made into a fermented milk of low viscosity as mentioned above by stirring the curd into pieces after the fermentation. On the contrary, with the methods of the present invention essentially including step (B-1), such a single curd bulk in the form of a contiguous gel is not generated, but curd pieces float, disperse, or precipitate in the whey. The size of the curd pieces may vary depending on various conditions and the kind of the lactic acid bacteria. For example, when the mixed material containing *Lactobacillus helveticus* CM4 is subjected alternately to the fermentation under stirring and the fermentation under static conditions, the size of the curd pieces will be about 3 μm to 5 mm.

[0028] In the present invention, the fermentation is preferably effected so that the growth of the lactic acid bacteria is not inhibited by excess oxygen, since the bacteria are facultative anaerobic. Accordingly, the stirring in the fermenting step is preferably carried out so that increase in the amount of oxygen is suppressed that is dissolved in the fermentation liquid due to entrainment of air bubbles therein. For example, the stirring, when continued all through the fermentation, is preferably carried out at low speed so that the fermentation liquid is softly mixed and fluidized. Specifically, the stirring speed may be about 1 to 50 rpm. Alternatively, when the fermentation is effected by a combination of fermentation under stirring and fermentation under static conditions, i.e., by a combination of steps (B-1) and (B-2), the stirring may be conducted vigorously for a short time to cause entrainment of air bubbles in the fermentation liquid, as long as increase in the amount of oxygen dissolved in the liquid is suppressed.

[0029] Surprisingly, by suitably selecting the above stirring conditions, the fermentation under stirring according to the present invention can provide fermented milk containing the ACEI peptide at the same ratio as or even higher ratio than the one produced only by the fermentation under static conditions, as demonstrated in the following Examples.

[0030] According to the method of the present invention, fermented milk that contains a large number of curd pieces and whey and that has a low viscosity and excellent workability, can be produced efficiently. Further, whey can also be produced efficiently from such fermented milk through the methods to be discussed later.

[0031] In the methods of the present invention, the fermenting steps may be followed by conventional stirring. In particular, when the fermentation includes step (B-2) of fermenting under static conditions, it is preferred to stir the fermentation product after termination of the fermentation.

[0032] The methods for producing whey containing an ACEI peptide of the present invention include, following the above production of the fermented milk, the step of subjecting the resulting fermented milk to centrifugation and/or filter pressing to separate and recover whey.

[0033] The centrifugation of the fermented milk may be carried out in a centrifuge. For example, it is preferred that

the centrifugation is carried out continuously at the revolution speed of about 2000 to 10000 rpm. The filter pressing may be carried out in a filter press. It is preferred that the filter pressing is carried out under the pressure of 2 to 8 kg/cm^2.

[0034] The fermented milk or whey containing an ACEI peptide obtained by the present methods may be used as fermented milk beverage or milk whey beverage. Further, the whey containing an ACEI peptide may be subjected to treatment such as deacidification, desaltation, concentration, isolation, and the like, for preparation of liquid products; or to drying and powdering treatments for preparation of products in the form of granules or tablets.

[0035] Since the methods for producing fermented milk containing an ACEI peptide of the present invention include fermentation under stirring, fermented milk with high ACEI peptide content can be produced efficiently. Further, the methods for producing whey containing an ACEI peptide of the present invention include the steps of fermenting under stirring to prepare fermented milk, and subjecting the resulting fermented milk to centrifugation and/or filter pressing to separate and recover whey. Thus, whey with high ACEI peptide content can be recovered efficiently. Therefore, these methods facilitate production of products containing an ACEI peptide, and are remarkably effective in industrial point of view.

[0036] The present invention will now be explained in detail with reference to Examples and Comparative Examples. However, the present invention is not limited to these.

Comparative Example 1

[0037] 900 g of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 9100 g of water, and the resulting solution was subjected to HTST (High Temperature Short Time) pasteurization at 90 °C for 1 minute. The pasteurized solution was cooled down to the room temperature, inoculated with 300 g of precultured *Lactobacillus helveticus* CM4, and stirred to make a uniform mixture. The mixture was then fermented under static conditions at 34 °C for 25 hours, to thereby obtain fermented milk curd (a) in the form of a contiguous gel with the lactic acid acidity of 2.06 wt %.

[0038] Next, the obtained fermented milk curd (a) was stirred and then placed in a centrifuge (manufactured by HITACHI LTD., 20PR52), which was operated at 3000 rpm for 10 minutes to remove curd fraction and recover 2.5 kg of whey.

[0039] The viscosity and ACEI peptide content of the fermented milk curd (a) were measured under the conditions below. The results are shown in Table 1. Further, the fermented milk curd (a) was stirred, and the particle size of the curd pieces was measured with a particle size analyzer (LA-920 manufactured by HORIBA LTD.). It was found that 90 % of the curd pieces had a diameter of not larger than 47 μm, and the arithmetic mean diameter was 27μm.

Viscosity Measurement

[0040] The viscosity was measured with VISMETRON viscometer (manufactured by SHIBAURA SYSTEM CO., LTD.) at the liquid temperature of 25 °C, revolution speed of 60 rpm, using rotor No. 2 for medium viscosity. The duration of measurement was 60 seconds.

Measurement of Val-Pro-Pro and Ile-Pro-Pro Contents

[0041] About 1 ml of fermented milk curd (a) as it was, was placed in an experimental centrifuge, which was operated at 15000 rpm for 10 minutes to collect the supernatant. 0.3 ml of the obtained supernatant was subjected to adsorption on Sep-Pak Cartrige (manufactured by WATERS CO.), followed by washing with distilled water. The adsorbed material was eluted with 5 ml of methanol, and dried in a centrifuging concentrator under reduced pressure. The obtained dried product was dissolved in 0.3 ml of a 0.05 % Trifluoroaceic acid aqueous solution, and analyzed by high performance liquid chromatography (HPLC).

[0042] Conditions of Analysis by HPLC

Apparatuses: HITACHI L4000UV DETECTOR
(detection at 215 nm)
L6200 Intelligent pump
L5030 Column Oven (35 °C)
Conditions of Isolation: Flow Rate at 0.5 ml/min.
Elution Solvent: 0.3 M NaCl, 0.05 % Trifluoroaceic acid aqueous solution
Column: Asahipak GS320 (Φ3.9×600 mm)
ACEI peptide Content: Content of ACEI peptides was calculated by the following formula since Val-Pro-Pro and Ile-Pro-Pro have different ACEI activities:

Content of ACEI peptides (mg/100g)

= Amount of IPP (mg/100g) × 1.7 + Amount of VPP (mg/100g)

Example 1

[0043] 900 g of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 9100 g of water, and the resulting solution was subjected to HTST pasteurization at 90 °C for 1 minute. The pasteurized solution was cooled down to the room temperature, inoculated with 300 g of precultured *Lactobacillus helveticus* CM4, and stirred to make a uniform mixture. The mixture was then fermented at 34 °C for 29 hours under stirring at 50 rpm, to thereby obtain fermented milk (b) with the lactic acid acidity of 1.88 wt%. The particle size of the curd pieces in the resulting fermented milk (b) was measured with the particle size analyzer (LA-920 manufactured by HORIBA LTD.). It was found that 90 % of the curd pieces had a diameter of not larger than 30 μm, and the arithmetic mean diameter was 18 μm.

[0044] Next, the obtained fermented milk (b) was placed in a centrifuge (manufactured by HITACHI LTD., 20PR52), which was operated at 3000 rpm for 10 minutes to remove curd fraction and recover 6 kg of whey.

[0045] The viscosity and ACEI peptide content of the fermented milk (b) were measured under the same conditions as in Comparative Example 1. The results are shown in Table 1. Incidentally, the viscosity was measured using rotor No. 1 for low viscosity, for the duration of 30 seconds.

Comparative Example 2

[0046] 1.5 kg of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 8.5 kg of water, and the resulting solution was subjected to HTST pasteurization at 90°C for 1 minute. The pasteurized solution was cooled down to the room temperature, inoculated with 300 g of precultured *Lactobacillus helveticus* CM4, and stirred to make a uniform mixture. The mixture was then fermented under static conditions at 34°C for 28 hours, to thereby obtain fermented milk curd (c) in the form of a contiguous gel with the lactic acid acidity of 2.81 wt%.

[0047] Next, the obtained fermented milk curd (c) was stirred and then placed in a centrifuge (manufactured by HITACHI LTD., 20PR52), which was operated at 3000 rpm for 10 minutes to remove curd fraction and recover 100 g of whey.

[0048] The viscosity and ACEI peptide content of the fermented milk curd (c) were measured under the same conditions as in Comparative Example 1. The results are shown in Table 1. Incidentally, the viscosity was measured using rotor No. 3 for high viscosity, for the duration of 60 seconds. The viscosity and ACEI peptide content of the fermented milk curd (c) were measured under the conditions below.

Example 2

[0049] 1.5 kg of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 8.5 kg of water, and the resulting solution was subjected to HTST pasteurization at 90 °C for 1 minute. The pasteurized solution was cooled down to the room temperature, inoculated with 300 g of precultured *Lactobacillus helveticus* CM4, and stirred to make a uniform mixture. The mixture was then fermented at 34 °C for 30 hours under stirring at 50 rpm, to thereby obtain fermented milk (d) with the lactic acid acidity of 3.04 wt%.

[0050] Next, the obtained fermented milk (d) was placed in a centrifuge (manufactured by HITACHI LTD., 20PR52), which was operated at 3000 rpm for 10 minutes to remove curd fraction and recover 6.4 kg of whey.

[0051] The viscosity and ACEI peptide content of the fermented milk (d) were measured under the same conditions as in Comparative Example 1. The results are shown in Table 1. Incidentally, the viscosity was measured using rotor No. 1 for low viscosity, for the duration of 30 seconds.

Example 3

[0052] 712 kg of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 7288 kg of water, and the resulting solution was subjected to plate pasteurization at 92 °C and then introduced into a tank (18000 liter tank manufactured by IWAI KIKAI). The pasteurized solution was cooled down to 35 °C, inoculated with 240 kg of precultured *Lactobacillus helveticus* CM4, and stirred to make a uniform mixture. The mixture was then fermented at 32 °C for 27 hours under intermittent stirring at 50 rpm (by repeating cycles of stirring for 15 minutes and leaving to stand for 45 minutes), to thereby obtain fermented milk (e) with the lactic acid acidity of 1.8 wt%. The particle size of the curd pieces in the resulting fermented milk (e) was measured with the particle size analyzer (LA-920 man-

ufactured by HORIBA LTD.). It was found that 90 % of the curd pieces had a diameter of not larger than 172 μm, and the arithmetic mean diameter was 86 μm.

[0053] Next, the obtained fermented milk (e) was placed in a nozzle separator (MBUX510T-34C manufactured by ALFALAVAL, nozzle size 1 mm, flow rate 3500 litter per hour), which was operated at 7490 rpm to recover 6160 kg of whey.

[0054] The viscosity and ACEI peptide content of the fermented milk (e) were measured under the same conditions as in Comparative Example 1. The results are shown in Table 1. Incidentally, the viscosity was measured using rotor No. 1 for low viscosity for the duration of 30 seconds.

Table 1

Fomented Milk	Viscosity (cp)	Whey Recovery (%)	ACEI Peptide Content (mg/100g)
Fermented Milk (a) (Comparative Example 1)	415	25	7.1
Fermented Milk (b) (Example 1)	4.5	60	9.0
Fermented Milk (c) (Comparative Example 2)	1832	1	10.5
Fermented Milk (d) (Example 2)	8.1	64	10.8
Fermented Milk (e) (Example 3)	3.8	77	8.6

Claims

1. A method for producing fermented milk containing an angiotensin converting enzyme inhibitory peptide comprising:

   (A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material, and
   (B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an angiotensin converting enzyme inhibitory peptide are generated,

   whereby fermented milk containing said curd pieces and said whey containing the angiotensin converting enzyme inhibitory peptide is produced.

2. A method for producing fermented milk containing an angiotensin converting enzyme inhibitory peptide comprising:

   (A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material,
   (B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an angiotensin converting enzyme inhibitory peptide are generated, and
   (B-2) fermenting said mixed material under static conditions,

   whereby fermented milk containing said curd pieces and said whey containing the angiotensin converting enzyme inhibitory peptide is produced.

3. The method of claim 1 or 2 wherein said milk is selected from the group consisting of cow's milk, goat's milk, sheep's milk, soy bean milk, skim milk, reconstituted milk, powdered milk, condensed milk and mixtures thereof.

4. The method of claim 1 or 2 wherein said fermented milk has a viscosity of not higher than 20 cp.

5. The method of claim 1 or 2 wherein said angiotensin converting enzyme inhibitory peptide is selected from the group consisting of Val-Pro-Pro, Ile-Pro-Pro, and mixtures thereof.

6. The method of claim 1 or 2 wherein said mixed material further contains a yeast.

7. The method of claim 1 or 2 wherein said lactic acid bacteria contained in the mixed material comprises *Lactobacillus helveticus*.

8. The method of claim 7 wherein said *Lactobacillus helveticus* comprises *Lactobacillus helveticus* CM4 (NATIONAL INSTITUTE OF BIOSCIENCE AND HUMAN TECHNOLOGY, AGENCY OF INDUSTRIAL SCIENCE AND TECHNOLOGY, Deposit No. FERM BP-6060, Deposit date: August 15, 1997).

9. A method for producing whey containing an angiotensin converting enzyme inhibitory peptide comprising:
subjecting the fermented milk produced by the method of claim 1 or 2 to at least one of centrifugation and filter pressing to separate and recover whey, is provided.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/00068

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] A23C9/123, A23C20/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] A23C9/12-9/15, A23C20/00-20/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, 583074, A1 (CALPIS FOOD IND CO LTD), 16 February, 1994 (16.02.94) & JP, 6-40944, A & US, 5449661, A & DE, 69326513, E & CN, 1090201, A	1-9
A	JP, 3-120225, A (AJINOMOTO CO., INC.), 22 May, 1991 (22.05.91) (Family: none)	1-9
A	JP, 7-75521, A (Asahi Chemical Industry Co., Ltd.), 20 March, 1995 (20.03.95) (Family: none)	1-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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Date of the actual completion of the international search	Date of mailing of the international search report
27 March, 2000 (27.03.00)	04 April, 2000 (04.04.00)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

PATENT ABSTRACTS OF JAPAN

(11)Publication number : 2001-163799
(43)Date of publication of application : 19.06.2001

(51)Int.Cl. A61K 38/00
A23L 1/305
A23L 2/52
A23L 2/66
A61K 7/00
A61K 7/48
A61K 9/08
A61P 17/00
A61P 17/16
A61P 27/02

(21)Application number : 11-349487
(22)Date of filing : 08.12.1999

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(54) LOW ANTIGENIC HUMECTANT, LOW ANTIGENIC EXTERNAL PREPARATION AND LOW ANTIGENIC BEVERAGE

(57)Abstract:
PROBLEM TO BE SOLVED: To obtain a low antigenic humectant, a low antigenic external preparation and a low antigenic beverage not inducing an anaphylactic reaction, having a high moisture retaining effect.
SOLUTION: This low antigenic humectant comprises ≥50 wt.% of a peptide composition having ≤ 20,000 molecular weight and an amino acid sequence of (Gly-X-Y)n obtained by specifically decomposing a gelatin component or a collagen component with a collagenase enzyme. A low antigenic external preparation of cosmetic, or the like, contains ≥0.005 wt.% of the low antigenic humectant.

(19)日本国特許庁（ＪＰ）　(12)公開特許公報（Ａ）　(11)特許出願公開番号
特開2001－163799
（P2001－163799A）
(43)公開日　平成13年６月19日(2001.6.19)

(51)Int.Cl.[7]	識別記号	ＦＩ		テーマコード(参考)
Ａ６１Ｋ　38/00		Ａ２３Ｌ	1/305	４Ｂ０１７
Ａ２３Ｌ　1/305		Ａ６１Ｋ	7/00	Ｃ　４Ｂ０１８
2/52				Ｊ　４Ｃ０７６
2/66			7/48	４Ｃ０８３
Ａ６１Ｋ　7/00			9/08	４Ｃ０８４

審査請求　未請求　請求項の数９　ＯＬ　（全８頁）　最終頁に続く

(21)出願番号　特願平11－349487

(22)出願日　平成11年12月８日(1999.12.8)

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(54)【発明の名称】　低抗原性保湿剤、低抗原性外用剤および低抗原性飲料

(57)【要約】
【課題】アナフィラキシー反応を誘発させず、かつ、高い保湿効果を有する低抗原性保湿剤、低抗原性外用剤および低抗原性飲料を提供する。
【解決手段】低抗原性保湿剤は、ゼラチン成分またはコラーゲン成分をコラゲナーゼ酵素を用いて特異的に分解して得られる、分子量が２０，０００以下であり、アミノ酸配列が（Ｇｌｙ－Ｘ－Ｙ）ｎのペプチド組成物を５０重量％以上含有する。化粧品その他の低抗原性外用剤は、低抗原性保湿剤を０．００５重量％以上含む。

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【特許請求の範囲】

【請求項１】ゼラチン成分またはコラーゲン成分をコラゲナーゼ酵素を用いて特異的に分解して得られる、分子量が２０，０００以下でアミノ酸配列が（Ｇｌｙ－Ｘ－Ｙ）ｎのペプチド組成物を５０重量%以上含有することを特徴とする低抗原性保湿剤。

【請求項２】ゼラチン成分またはコラーゲン成分をコラゲナーゼ酵素を用いて特異的に分解して得られる、分子量が２０，０００以下でアミノ酸配列が（Ｇｌｙ－Ｘ－Ｙ）ｎのペプチド組成物をリポソーム化したものを５０重量%以上含有することを特徴とする低抗原性保湿剤。

【請求項３】ゼラチン成分またはコラーゲン成分をコラゲナーゼ酵素を用いて特異的に分解して得られる、分子量が２０，０００以下でアミノ酸配列が（Ｇｌｙ－Ｘ－Ｙ）ｎのペプチド組成物をマイクロカプセル化したものを５０重量%以上含有することを特徴とする低抗原性保湿剤。

【請求項４】前記ペプチド組成物はｎ＝１，２または３のものを５重量%以上含有することを特徴とする請求項１，２または３記載の低抗原性保湿剤。

【請求項５】請求項１，２，３または４記載の低抗原性保湿剤を０．００５重量%以上含むことを特徴とする低抗原性外用剤。

【請求項６】アトピー性皮膚炎用、ドライスキン用、ベビー用、敏感肌用または介護用のスキンケア用外用剤であることを特徴とする請求項５記載の低抗原性外用剤。

【請求項７】化粧品であることを特徴とする請求項５記載の低抗原性外用剤。

【請求項８】ドライアイ用点眼剤であることを特徴とする請求項５記載の低抗原性外用剤。

【請求項９】請求項１，２，３または４記載の低抗原性保湿剤を０．００５重量%以上含むことを特徴とする低抗原性飲料。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は、ゼラチン成分またはコラーゲン成分をコラゲナーゼ酵素を用いて特異的に分解して得られる、アナフィラキシー反応を誘発させない低抗原性保湿剤、低抗原性外用剤および低抗原性飲料に関する。

【０００２】

【従来の技術】人体を清潔に保つため、または、美しく装うために、基礎化粧品、メーキャップ化粧品、毛髪化粧品、ボディ化粧品など種々の化粧品や医薬部外品が開発されており、これらの多くに主に保湿剤や系の安定化剤として、コラーゲンやゼラチンあるいはそれらを酸や加熱によって処理した分解物が用いられている。コラーゲン分子を構成しているアミノ酸は、親水性の側鎖をもつものが多く、水分子をたくさん吸着できるため保湿効果が高く、肌へのなじみも良く、また、アレルギー反応

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を引き起こし難いという経験に基づいて広く使用されるようになった。

【０００３】

【発明が解決しようとする課題】一方、文明病といわれているアレルギー疾患の患者数は近年欧米各国を始め、日本国内でも急激に増加の一途をたどっており、現在では実に３人に１人の割合で何らかのアレルギー疾患をもっているとさえ言われている。このようなアレルギー患者数の増加を背景にして、抗原性／アレルゲン性がほとんどないと考えられていたゼラチンやコラーゲンに対してもアナフィラキシーなどの副作用を引き起こす（ゼラチン特異ＩｇＥ抗体をもった）患者が最近少しずつ増えてきている為、社会問題化し始めいている。事実、これらに関する学術的な発表は１９９０年代に入ってから初めて散見されるようになってきた（Kelso,J.M.等，J.Allergy Clin.Immunol.，91巻，867－872頁、Sakaguchi，M.等,感染・炎症・免疫,26巻，48－50頁参照）。

【０００４】コラーゲンやゼラチンの他に保湿剤として一般的に用いられているものとしては、グリセリン、プロピレングリコールなどの多価アルコール類、尿素、ラノリン、ヒアルロン酸、ビタミンＥなどがあり、それらに加えて最近ではセラミドも注目されているが、多価アルコールでは、べたつきといった不快感があり、尿素は浸透圧が高いことに起因すると思われる刺激性、ラノリンでは接触皮膚炎などの副作用が報告されている。

【０００５】さらに近年、乾燥、紫外線、ストレスなどの外的要因や加齢による皮膚の老化などの内的要因によりドライスキン、敏感肌、肌荒れ、アトピー性皮膚炎などが増加しており、これらが皮膚保湿と密接に関わっていることから、より安全な保湿剤の開発が求められていた。

【０００６】そこで、本発明者らは、前記のような実情に鑑みて、抗原性やアレルゲン性を示さないゼラチンあるいはコラーゲンの誘導体について鋭意研究を重ねた。その結果、コラゲナーゼ酵素を単独ないしは各種の担体に固定化させた状態で、ゼラチン成分ないしはコラーゲン成分を含む原材料に直接作用させ、特異的な酵素分解を行わせることで、抗原性がなく、コラーゲン本来の特徴的なアミノ酸配列である（Ｇｌｙ－Ｘ－Ｙ）ｎを保持した分子量範囲が 1,000 以下のペプチド組成物が高収率で得られることを見出した（特開平7-82299号公報）。

【０００７】さらに、本発明者らは、鋭意研究を重ねた結果、上記製造工程に若干の工夫を加えることで、分子量 1,000 以下のペプチドだけでなく、抗原性／アレルゲン性を実質的に示さない、分子量約 1,000 ～ 20,000 の範囲にあるペプチド組成物も作成可能であることを見出した。この低抗原性ペプチド組成物は、従来のゼラチンやコラーゲンおよびそれらの分解物と同様に、各種生理活性物質および生物活性物質を安定化させる作用が

あり（特開平9-176196号公報、特開平11-12196号号公報）、特にワクチンの安定化剤として実用段階に至っている。

【０００８】しかしながら、従来、コラーゲンやゼラチンの保湿効果は、低分子化が進むにつれ弱くなると考えられており、本発明者らによる特開平7-82299号公報、特開平9-176196号公報および特開平11-12196号公報に示す分子量が２０，０００以下のペプチド組成物に保湿剤としての用途は考えられなかった。

【０００９】本発明は、このような問題点を解決するためになされたもので、アナフィラキシー反応を誘発させず、かつ、高い保湿効果を有する低抗原性保湿剤、低抗原性外用剤および低抗原性飲料を提供することを目的としている。

【００１０】

【課題を解決するための手段】本発明者らは、ゼラチン成分あるいはコラーゲン成分をコラゲナーゼ酵素を用いて特異的に分解して得られるペプチド組成物は、分子量が２０，０００以下のものでも、抗原性／アレルゲン性を実質的に示さないだけでなく、高い保湿効果を有することを見出した。

【００１１】そこで、本発明に係る低抗原性保湿剤は、ゼラチン成分またはコラーゲン成分をコラゲナーゼ酵素を用いて特異的に分解して得られる、分子量が２０，０００以下でアミノ酸配列が（Ｇｌｙ－Ｘ－Ｙ）ｎのペプチド組成物を５０重量%以上含有することを特徴とする。アミノ酸配列の（Ｇｌｙ－Ｘ－Ｙ）ｎの式で、ＸおよびＹはＧｌｙ以外の任意のアミノ酸残基、例えば、ＰｒｏやＨｙｐであり、ｎは自然数である。

【００１２】本発明に係る低抗原性保湿剤は、前記ペプチド組成物をリポソーム化したものを５０重量%以上含有するものであってもよい。また、本発明に係る低抗原性保湿剤は、前記ペプチド組成物をマイクロカプセル化したものを５０重量%以上含有するものであってもよい。本発明に係る低抗原性保湿剤では、前記ペプチド組成物はｎ＝１，２または3のものを５重量%以上含有することが好ましい。

【００１３】本発明に係る低抗原性外用剤は、前記低抗原性保湿剤を０．００５重量%以上含むことを特徴とする。本発明に係る低抗原性外用剤は、アトピー性皮膚炎用、ドライスキン（乾皮症）用、ベビー用、敏感肌用または介護用のスキンケア用外用剤であっても、化粧品であっても、ドライアイ用点眼剤であってもよい。本発明に係る低抗原性外用剤は、医薬部外品であってもよい。化粧品としては、清浄用化粧品、頭髪化粧品、基礎化粧品、メークアップ化粧品、芳香化粧品、日焼け・日焼け止め化粧品、爪化粧品、アイライナー化粧品、口唇化粧品、口腔化粧品、入浴用化粧品などが挙げられ、より具体的には、化粧水、乳液、美容液、パック、ファンデーション、口紅、石鹸、ハンドクリーム、シャンプー、リンス、トリートメントなどが挙げられる。医薬部外品としては、例えば、ドライアイ用点眼剤のほか、薬用化粧品、育毛剤などが挙げられる。

【００１４】本発明に係る低抗原性保湿剤は、べたつかず、のびが良く、皮膚への浸透性が高く、皮膚の保湿力を向上させる。本発明に係る低抗原性外用剤は、保湿力の低下した肌あるいは眼の保湿力向上に有用である。

【００１５】本発明に係る低抗原性飲料は、前記低抗原性保湿剤を０．００５重量%以上含むことを特徴とする。低抗原性飲料としては、例えば、栄養ドリンクが挙げられる。本発明に係る低抗原性飲料は、口内やのどの保湿力を向上させ、効果的に潤す。

【００１６】本発明に係る低抗原性保湿剤の出発原材料であるゼラチン成分またはコラーゲン成分は、牛、豚、鳥、鯨などの動物の骨、皮、腱、または鮫などの魚皮を原料として調製することができる。コラゲナーゼ酵素には、Clostridium histolyticum, Streptomyces parvulusなどの細菌、放線菌あるいは真菌など由来で、コラーゲン特有のアミノ酸配列：（Gly-X-Y）ｎのグリシンのアミノ基側を特異的に切断する酵素を用いる。また、これらの酵素遺伝子を遺伝子工学的に特定のベクターに組み込んで、乳酸菌や酵母などの他の菌体あるいは動物に産生させて得られた遺伝子組み替えによる酵素で、類似の基質特異性を有するコラーゲン酵素を用いてもよい。

【００１７】本発明におけるコラゲナーゼ酵素を使用する際に特に留意すべき点は、コラゲナーゼ酵素の純度である。通常、各種菌体から調製されたコラゲナーゼ酵素には、他の蛋白質分解酵素（プロテアーゼ）が混入していることがある。この不純酵素が多く含まれると原材料中のゼラチン成分あるいはコラーゲン成分以外の蛋白質も分解されてしまったり、ゼラチンあるいはコラーゲン成分自身もその不純酵素によって非特異的に分解されてしまうため、精製されてくるペプチド組成物の品質が低下してしまう。ひいては、アナフィラキシーを誘発させる原因となる可能性が高い。従って、使用するコラゲナーゼ酵素の純度については、その基質特異性と共に十分注意を払う必要がある。

【００１８】コラゲナーゼ酵素は、遊離の形で使用しても良いし、コラゲナーゼ酵素を物理吸着法あるいは化学結合法によって各種の担体に結合させた固定化酵素として使用しても良い。また、コラゲナーゼ酵素による酵素分解の方法としては、(a)バッチ法、(b)カラム法あるいは(c)これらを組み合わせた方法などを用いることができる。

【００１９】これら（ａ）～（ｃ）の方法による製造ラインと、使用するコラゲナーゼ酵素の形態とは、それぞれ自由に組み合わせることができる。本発明に係る低抗原性保湿剤の製造は、特開平7-82299 号に記載の方法に準じて実施することができるが、他の方法でも可能である。むしろ、原料となるゼラチン成分やコラーゲン成分

に応じた方法を選択したり、コラゲナーゼ酵素の特異性や純度などを維持するのに適した方法を選択することが好ましい。

【００２０】より具体的に、本発明に係る低抗原性保湿剤の製造方法の一例を挙げれば、以下の実施例で示すように、ゼラチンあるいはコラーゲンを出発原料とし、酵素の回収を良くするために固定化コラゲナーゼ酵素を用いたバッチ法やカラム法によるバイオリアクター方式で製造することができる。すなわち、コラゲナーゼ酵素を物理吸着法あるいは化学結合法によって各種の担体、例えばキトパールなどに結合させ、クロマトグラフィー用のカラムに充填した通常のシステムに、一定温度下、可溶化させたゼラチン溶液あるいは分解が生じない程度の温度、望ましくは 40 ～ 45℃で変性させたコラーゲンを通して酵素分解をさせる。この際のコラーゲン原料の送液速度は、固定化酵素の活性および必要とされる分解度合いに応じて適宜選択される。

【００２１】以下、実施例により本発明を詳細に説明するが、これらの実施例は、本発明の範囲を何ら制限するものではない。また、本発明によるところのゼラチンやコラーゲン由来のペプチド組成物を保湿剤として使用する方法は、すでに公知となっている操作方法に準じて実施することができる。

【００２２】

【実施例１】高純度ゼラチン（宮城化学工業社製） 50 gを 1,000 mlの 20 mM Tris-HCl緩衝液（pH 7.4）／0.1 M NaCl に加温しながら溶解後、50℃に冷却した。酵素分解用の固定化酵素は、100 mg のコラゲナーゼ酵素（ワシントン社製、typeⅠⅤから精製した高純度品）を 50 g のキトパール（富士紡績社製）に２架橋試薬を用いて結合させて調製した。担体への結合量は結合前後の 280 nm における吸光度の変化を計測して算出したが、９９%以上の結合率であった。使用時、本固定化酵素をカラムとカラムの間に pH センサーを設置した２連式の＊カラム式バイオリアクターに充填し、20 mM Tris-HCl 緩衝液（pH 7.4）／0.1M NaCl 緩衝液で良く洗浄・平衡化を行った。 pH の測定システムは、この２連式カラムのカラム間に設置した pHセンサーが変化を感知して、繋いであるチューブから濃厚 Tris 緩衝液が流入するシステムとなっている。

【００２３】上記工程で得られた高純度コラーゲンを、上記工程で調製された縦型２連式のコラゲナーゼ酵素固定化カラムにアプライし、カラム法による酵素分解を行った。この間、流速は毎分 50 ～ 80 ml に、またカラムの温度は 39 ± 1 ℃にコントロールした。最終（２連目）のカラムから出てきた酵素反応終了液を分取し、0.45 μm のフィルターで濾過を行った。

【００２４】この濾液をスプレードライ（噴霧乾燥器）で粉末化した後、ゲル濾過器（商品名：スーパーデックス 30pg、ファルマシア社製）を用いたゲル濾過法、あるいは ODS カラム（ワイエムシー社製）を用いた逆相クロマトグラフィー法によって、分子量 1,000 以下あるいは分子量 約 20,000 以下のペプチド組成物をそれぞれ精製した。これらのペプチド組成物は、本実施例１による低抗原性保湿剤である。

【００２５】上記工程で得られた各ペプチド組成物の分子量分布を高速液体クロマトグラフィー：ＨＰＬＣ（島津製作所社製 LC-10A、カラム：ｽｰﾊﾟｰﾃﾞｯｸｽ peptide）で測定した。各試料は、HPLCカラムへインジェクションする前に 0.2 μm のメンブレンフィルターで濾過を行った。実施例１から得られた各ペプチド組成物、すなわち、分子量 約 20,000 以下のペプチド画分および分子量 1,000 以下のペプチド画分について、平均分子量、分子量範囲、分子量 500 以下のペプチドの割合を表１にまとめた。

【００２６】

【表１】

ペプチド組成物	平均分子量 M.W.	≦1,000 のペプチド (%)	≦ 500 のペプチド (%)
≦ 20,000	3,200	15.2 %	5.6 %
≦ 1,000	530	99.8 %	52.2 %

【００２７】上記工程で得られた各ペプチド組成物、すなわち、分子量 約 20,000 以下のペプチド組成物および分子量 1,000 以下のペプチド組成物を凍結乾燥した後、それぞれの NH_2 末端アミノ酸および NH_2 末端から２番目のアミノ酸についてエドマン分解法によって検定した。その結果、両者の NH_2 末端側のアミノ酸はそれぞれ 95.7%および 97.5%がグリシンであることが判明し、本発明によるところのコラーゲンに特徴的なアミノ酸配列である (Gly-X-Y)n 構造を保持していることが証明された。上記工程で得られた各ペプチド組成物は、低抗原性保湿剤として用いることができる。

【００２８】上記工程で得られたペプチド組成物（低抗原性保湿剤）の抗原性／アレルゲン性の消失に関する検定方法およびその結果について示す。

【００２９】〔ゼラチン抗血清（ＩｇＧタイプ）の調製〕牛皮および豚皮由来のゼラチンをＰＢＳに溶解して２ｍｇ／ｍｌとし、0.22μｍのフィルターで濾過を行った溶液をフロイント完全アジュバントと等量づつ混和してエマルジョンを調製し、３羽のウサギに１ｍｌずつ注射した。その３週間後、同じペプチド組成物の溶液を等

量のフロイント不完全アジュバントと共に混和してエマルジョンを作成し、同様にウサギに注射した。この操作を３回繰り返し、最終免疫後１週間目に抗血清を得た。

【００３０】〔ゼラチン抗血清（ＩｇＥタイプ）の調製〕牛皮および豚皮由来のゼラチンをＰＢＳに溶解して２ｍｇ／ｍｌとし、0.22μmのフィルターで濾過滅菌を行った溶液に水酸化アルミニウム（Alum）で沈澱させ、洗浄したもの 100μg/ml に調製し、３羽のモルモットの皮内に１ｍｌずつ注射した。その４週間後、同様にして追加免疫を行った３～５日後に抗血清を得た。

【００３１】〔ゼラチン感作イムノボールの調製〕２架橋試薬で活性化されたアミノ化ポリスチレンボール（住友ベークライト社製）の官能基（NH_2 基）に牛皮あるいは豚皮由来のゼラチンを固定化させ、牛血清アルブミンあるいは界面活性剤などでブロッキング操作を行ったゼラチン感作イムノボールを作成した。

【００３２】

【試験１】〔酵素免疫測定法（抗原性試験－１）〕（阻害反応による抗原性の検定）ゼラチン感作イムノボールに、（１）実施例１で得られた分子量約20,000以下のペプチド組成物（実施例１の低抗原性保湿剤）、（２）実施例１で得られた分子量1,000 以下のペプチド（実施例１の低抗原性保湿剤）、（３）加熱分解して得られた分子量200 ～7,000 の部分分解ゼラチン（比較＊例）、（４）トリプシンとペプシンで酵素分解させた分子量500 ～12,000の酵素分解ゼラチン（比較例）および（５）ゼラチン（比較例）、を各200 μl ずつ添加し、次いで、前記ＩｇＧタイプのウサギゼラチン抗血清あるいはＩｇＥタイプのモルモットゼラチン抗血清のいずれかを 200μl加えて 37℃で 30 分間反応させて、抗血清とゼラチン抗原との反応系における（１）～（５）各成分の競合反応を行わせた。

【００３３】次いで、洗浄し、山羊抗ウサギＩｇＧ抗体の西洋ワサビパーオキシダーゼ（ＨＲＰ）標識複合体（コスモ・バイオ）あるいは山羊抗モルモットＩｇＥ抗体の西洋ワサビパーオキシダーゼ（ＨＲＰ）標識複合体（コスモ・バイオ）を２次反応させた。３７℃で１時間反応後、イムノボールに結合して残っているＨＲＰ標識複合体の活性を測定することによって、（１）～（５）各成分の抗原性およびアレルゲン性をその競合反応の程度から検討した。その結果を表２に示す。表２の結果から、（１）および（２）の実施例１の低抗原性保湿剤は、阻害率が０％であり、（３）～（５）の比較例のものが抗原性を有するのに対して抗原性を有しないことがわかる。

【００３４】

【表２】

試　料（処理方法）	分 子 量	阻　害　率（％）
①本発明のペプチド	≦ 20,000	0　％
②本発明のペプチド	≦ 1,000	0
③加熱分解ゼラチン	200 ～ 7,000	8．1
④ トリプシン／ペプシン 酵素分解ゼラチン	500 ～ 12,000	0．6
⑤ゼラチン	(-)	１００

【００３５】

【試験２】〔受身皮膚アナフィラキシー(Passive Cutaneous Anapylaxis:PCA)（抗原性試験２）〕滅菌生理食塩水を用い、モルモット抗牛ゼラチン抗血清の 1/2 希釈系列（1/10, 1/20, 1/40, 1/80, 1/160）を作成し、各々の各希釈血清 50 μl を背毛を刈ったＳＤ系ラット（オス、8週齢）２匹ずつ（計４匹）の背部皮内に注射した。 24 時間後、このうちの１匹に、実施例１で得られた分子量約20,000以下のペプチド組成物（実施例１の低抗原性保湿剤）の １mg を含む 0.6 % エバンスブルー溶液 1.0 ml を尾静脈より注射した。また、ペプチド組成物に対する陽性コントロールとして、２匹目のＳＤ系ラットにも同様に牛ゼラチンの １mg を含む0.6 % エバンスブルー溶液 1.0 ml を尾静脈より注射した。60 分後４匹とも屠殺し、背部皮膚を剥いで紫斑を観察し、それらの大きさを測定した。

【００３６】判定は、紫斑径が 10 mm 以上を（＋＋）または（＋＋＋）、9 mm ～ 5 mmを（＋）、4 mm ～ 1 mm を（±）とし、紫斑が生じない場合を（－）とした。その結果を表３に示す。表３の結果から、牛ゼラチンの場合、１／160 の希釈系列でも紫斑が生じるのに対し、実施例１で得られた分子量約20,000以下のペプチド組成物（低抗原性保湿剤）の場合には、１／10の希釈系列でも紫斑が生じず、抗原性を有しないことがわかる。

【００３７】

【表３】

試　料	抗血清 希釈系列	ＰＣＡ反応の判定結果
牛ゼラチン	1/ 10 1/ 20 1/ 40 1/ 80 1/160	(＋＋～＋＋＋) (＋＋) (＋) (＋) (±)
本発明のペプチド	1/ 10 1/ 20 1/ 40 1/ 80 1/160	(－) (－) (－) (－) (－)

【００３８】
【試験３】〔蛍光酵素免疫測定法（抗原性試験３）〕（阻害反応によるアレルゲン性の検定）ゼラチンに対してアレルギー症状を呈している患者６名（表４中の患者Ａ～Ｆ）から採血された血清および前述のゼラチン感作イムノボールを用いて、ゼラチン特異ＩｇＥ抗体を測定する蛍光酵素免疫測定法を実施する際に、蛍光酵素免疫測定法で陽性となった患者の特異ＩｇＥ抗体の力価が実施例１のペプチド組成物（低抗原性保湿剤）によってどの程度阻害されるかについて、標識酵素によって分解された蛍光基質の蛍光強度を測定することによって判定した。なお、ゼラチン特異ＩｇＥ抗体を検出するための第２抗体としてはマウス抗ヒトＩｇＥ抗体のβガラクトシダーゼ標識体を使用し、その酵素活性は蛍光基質を用いて検定した。

＊【００３９】その結果、表４に示されるように，実施例１で得られた分子量約20,000以下のペプチドおよび分子量1,000 以下のペプチド（実施例１の低抗原性保湿剤）の両成分とも阻害反応による抗体力価（蛍光強度）の低下が観察されず、ゼラチン特異ＩｇＥ抗体とは全く反応性を有しないことが判明した。これに対し、もとの原料であるゼラチン、加熱分解ゼラチンあるいは非特異的なプロテアーゼで分解したゼラチンでは強い阻害が生じ、ゼラチン特異ＩｇＥ抗体と良く反応性することが示され、アナフィラキシーが原因の一端が明らかになった。この試験３を行うことによって、Ｉ型アレルギーを誘導するゼラチン特異ＩｇＥと本ペプチド組成物（低抗原性保湿剤）が反応性をもっているかを知ることができる。

【００４０】
＊【表４】

試　料		各アレルギー患者の抗体力価 *1					
試料名	分子量	A*1	B*1	C*1	D*1	E*1	F*1
対照群：試料なし	(-)	8,890	1,542	9,004	8,863	1,034	8,226
本発明のﾍﾟﾌﾟﾁﾄﾞ	≦ 20,000	8,778	1,606	8,905	8,660	1,029	8,007
本発明のﾍﾟﾌﾟﾁﾄﾞ	≦ 1,000	8,996	1,621	9,660	8,696	1,160	8,644
ｾﾞﾗﾁﾝ	(-)	257	89	822	85	41	307
加熱分解ｾﾞﾗﾁﾝ	200～ 7,000	584	115	978	1,601	(-)	(-)
ﾄﾘﾌﾟｼﾝ／ｷﾓﾄﾘﾌﾟｼﾝ 酵素分解ｾﾞﾗﾁﾝ	500～12,000	1,007	105	655	1,025	(-)	(-)

*1：蛍光強度（F.I.）で表示した。

【００４１】
【試験４】〔経皮吸収試験（ミクロオートラジオグラフィーによる検討）〕実施例１による分子量約20,000以下のペプチド組成物（低抗原性保湿剤）を^{3}Ｈで標識したものを１０ｍｇ精秤し、これにプロピレングリコール、グリセリン、ＰＥＧ１５００を重量比６：４：５で混合した液０．３ｇに、精製水１．４９ｇおよびエタノール０．２ｇを加えて経皮投与製剤を調製した。この経皮投与製剤を、前日にエーテル麻酔下で剃毛したラットの背部中央に少量ずつ滴下しながらスパーテルで塗布した。摘出した皮膚を用いてミクロオートラジオグラムを作製し、皮膚中の放射能分布を調べたところ、角質層、顆粒層、有棘層、基底層のいたるところに観察され、さらには、真皮にまで到達している様子が観察された。これにより、実施例１のペプチド組成物（低抗原性保湿剤）の経皮吸収性が高いことが確認された。

【００４２】
【配合例１】〔化粧水〕実施例１による分子量約20,000以下のペプチド組成物（低抗原性保湿剤）０．７７ｇおよびＰＥＧ１５００の７．７ｇを精製水５０ｍｌに溶解した後、プロピレングリコール９．２４ｇおよびグリセリン６．１６ｇを加えよく混和した。これに、パラベン０．１５４ｇをエタノール１５．４ｍｌに溶解させたものを添加し、精製水を加えて全量を１５４ｍｌとし、さらによく混和した。これにより、保湿性が高く、使用時ののびもよくべたつかない低抗原性の化粧水が得られた。

【００４３】

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【配合例２】〔乳液〕プロピレングリコール５．０ｇ、ＰＥＧ３．０ｇ、トリエタノールアミン１．０ｇを精製水６０ｍｌに加え、７０℃に加熱調整して、水相を準備した。ステアリン酸２．０ｇ、セチルアルコール１．０ｇ、ワセリン２．０ｇ、スクワラン５．０ｇ、グリセロールトリ－２－エチルヘキサン酸エステル２．０ｇを７５℃で溶解し、これにソルビタンモノオレイン酸エステル２．０ｇ、パラベン０．１ｇを加え、７０℃に調整した。この油相を先に調整した水相に加え、さらに、実施例１による分子量約20,000以下のペプチド組成物をリポソーム化したペプチド組成物（低抗原性保湿剤）（０．２ｇリン脂質／ｍｌ）１５ｍｌを加えてよく撹拌混合した後、ホモミキサーで乳化粒子を均一にし、脱気、濾過、冷却を行った。これにより、保湿性が高く、使用時ののびもよくべたつかない低抗原性の乳液が得られた。

【００４４】

【配合例３】〔保湿美容液〕実施例１による分子量約20,000以下のペプチド組成物（低抗原性保湿剤）５．０ｇ、ソルビトール８．０ｇ、プロピレングリコール５．０ｇ、ＰＥＧ１５００の７．０ｇを精製水６６．７ｍｌに溶解した。エタノール７．０ｇにＰＯＥオレイルアルコールエーテル１．０ｇ、オリーブ油０．２ｇ、パラベン０．１ｇを溶解し、配合例２の水相を加えて溶解した。これにより、保湿性が高く、使用時ののびもよくべたつかない低抗原性の保湿美容液が得られた。

【００４５】

【配合例４】〔ハンドクリーム〕実施例１による分子量約20,000以下のペプチド組成物（低抗原性保湿剤）０．５ｇ、グリセリン１５．０ｇ、プロピレングリコール３．０ｇ、水酸化カリウム０．２ｇを精製水６６．２＊ｍｌに、７０℃に加熱調整して、水相を準備した。ステアリン酸３．０ｇ、ステアリン酸モノグリセリド３．０ｇ、ワセリン３．０ｇ、流動パラフィン６．０ｇ、パラベン０．１ｇを加え、７０℃に調整した。この油相を先に調整した水相に加え、よく撹拌混合した後、ホモミキサーで乳化粒子を均一にし、脱気、濾過、冷却を行った。これにより、保湿性が高く、使用時ののびもよくべたつかない低抗原性のハンドクリームが得られた。

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【００４６】

【配合例５】〔ドライスキン用化粧水〕実施例１による分子量約20,000以下のペプチド組成物（低抗原性保湿剤）２．０ｇを精製水５０ｍｌに溶解し、これにパラベン０．１ｇをエタノール１０．０ｍｌに溶解させたものを添加した。さらに、精製水を加えて全量を１００ｍｌとし、よく混和した。これにより、保湿性が高く、使用時ののびもよくべたつかない低抗原性のドライスキン用化粧水が得られた。

【００４７】

【配合例６】〔ドライアイ用点眼剤〕実施例１による分子量約20,000以下のペプチド組成物（低抗原性保湿剤）０．５ｇを精製水５０ｍｌに溶解し、これにパラベン０．０２ｇをエタノール ２．０ｍｌに溶解させたものを添加した。さらに、精製水を加えて全量を１００ｍｌとし、よく混和した。これにより、保湿力の高い低抗原性のドライアイ用点眼剤が得られた。

【００４８】

【発明の効果】本発明の低抗原性保湿剤、低抗原性外用剤および低抗原性飲料によれば、抗原性／アレルゲン性を示さないだけでなく、使用感、皮膚への浸透性、保湿性に優れる。

フロントページの続き

(51)Int.Cl.⁷		識別記号	FI		テーマコード（参考）
Ａ６１Ｋ	7/00		Ａ６１Ｐ	17/00	
	7/48			17/16	
	9/08			27/02	
Ａ６１Ｐ	17/00		Ａ６１Ｋ	37/18	
	17/16		Ａ２３Ｌ	2/00	Ｆ
	27/02				Ｊ

Ｆターム(参考) 4B017 LC03 LK15 LK23 LL09 LP06
4B018 LB08 MD20 MD22 MD69 MD90
ME07 MF12
4C076 AA12 BB24 CC10 DD37 EE42
EE43 FF57
4C083 AA122 AB032 AC012 AC022
AC072 AC102 AC122 AC132
AC182 AC242 AC422 AC442
AC482 AC542 AD042 AD411
AD412 AD431 AD432 CC04
CC05 DD23 DD31 EE12
4C084 AA02 BA02 BA43 BA44 MA58
MA63 ZA891 ZA892 ZB131
ZB132

(WO/2000/041572) PROCESS FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND PROCESS FOR PRODUCING MILK SERUM

Latest bibliographic data on file with the International Bureau

Publication Number: WO/2000/041572
Publication Date: 20.07.2000
Chapter 2 Demand Filed: 08.08.2000
International Application No.: PCT/JP2000/000068
International Filing Date: 11.01.2000

Int. Class.: ***A23C 19/045*** (2006.01), ***A23C 19/05*** (2006.01), ***A23C 21/00*** (2006.01), ***A23C 9/123*** (2006.01)

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Priority Data: 11/3946 11.01.1999 JP

Title: PROCESS FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND PROCESS FOR PRODUCING MILK SERUM

Abstract: Processes for producing fermented milk and milk serum whereby fermented milk and milk serum containing an ACEI peptide at a high content, which have high safety and are usable as drugs, funtional foods, health foods, etc., can be efficiently obtained at a high yield. A process for preparing fermented milk containing curd pieces and milk serum containing an angiotensin converting enzyme inhibitory peptide which involves the step of mixing and stirring a milk-containing material with lactic acid bacteria to give a mixed material, and the step of fermenting the mixed material under stirring so as to form the milk serum containing the angiotensin converting enzyme inhibitory peptide; and a process for producing milk serum further involving the step of centrifuging and/or compression-filtering the fermented milk obtained above to thereby separate and collect the milk serum.

Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
African Regional Intellectual Property Org. (ARIPO) (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW)
Eurasian Patent Organization (EAPO) (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM)
European Patent Office (EPO) (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE)
African Intellectual Property Organization (OAPI) (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Publication Language: Japanese (JA)

Filing Language: Japanese (JA)

JP1095736

Title:
PRODUCTION OF RICE CAKE-LIKE FOOD AND DEVICE THEREFOR

Abstract:

PURPOSE:To contrive to produce a food for rice cake close to handmade rice cake pounded by a mortar and a pestle, by using a twin-screw extruder having a heating and a blending zones and adding a blowing process to introduce a gas in the material at the early stage of the blending zone. CONSTITUTION:Glutinous rice or powder thereof is fed to a twin-screw extruder 2 and necessarily hydrated and a food for rice cake is continuously, extruded through a heating and steaming or boiling zone B while transferring in a barrel 1, a heating and humidifying zone C similarly and a blending and blowing zone D while transferring from an outlet. A blowing process to introduce a gas into a material under pressure by a nozzle 11 at the early stage of the blending zone is added to the process. Consequently, a high-quality food for rice cake having a taste close to that of a handmade rice cake pounded by a mortar and a pestle can be rapidly prepared.

Search result: 1 of 1

(WO/1998/005343) ANTI-STRESS DRUGS AND FUNCTIONAL FOODS HAVING ANTI-STRESS EFFECTS

Biblio. Data | Description | Claims | National Phase | Notices | Documents

Latest published bibliographic data

Publication No.: WO/1998/005343
Publication Date: 12.02.1998
International Application No. PCT/JP1997/002728
International Filing Date: 06.08.1997

Int. Class.[8]: ***A23C 9/123, A23C 9/137, A23L 1/30, A61K 35/20, A61K 35/74.***

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Priority Data: 8/208504 07.08.1996 JP

Title: (EN) ANTI-STRESS DRUGS AND FUNCTIONAL FOODS HAVING ANTI-STRESS EFFECTS
(FR) MEDICAMENTS ANTI-STRESS ET ALIMENTS FONCTIONNELS AYANT DES EFFETS ANTI-STRESS

Abstract: (EN) Anti-stress drugs which can be continuously administered without any problem in safety and relieve or prevent mental and physical symptoms caused by stresses and functional foods having anti-stress effects. The drugs contain as the active ingredient acid milks prepared by fermenting animal milks by using lactic acid bacteria belonging to the genus $i(Lactobacillus), which the functional foods contain these drugs and have anti-stress effects.

(FR) Cette invention se rapporte à des médicaments anti-stress qui peuvent être administrés en continu sans problème de sécurité et qui soulagent ou préviennent les symptômes mentaux et physiques causés par le stress, ainsi qu'à des aliments fonctionnels ayant des effets anti-stress. Ces médicaments contiennent comme principe actif des laits acides préparés par fermentation de laits d'animaux au moyen de bactéries d'acide lactique appartenant au genre $i(Lactobacillus), ces médicaments étant contenus dans les aliments fonctionnels objets de cette invention qui ont des effets anti-stress.

Designated States: AU, BG, BR, CA, CN, CZ, HU, KR, MX, PL, RO, SG, SI, SK, TR, US.
European Patent Office (EPO) (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE)

Publication Language: Japanese (JA)

Filing Language: Japanese (JA)

JP1045610

Title:
BOAT-FORM TUB-SHAPED PLASTIC SANITARY BODY AND MANUFACTURE AND DEVICE THEREOF

Abstract:

PURPOSE: To improve the adiabatic efficiency and provide a product of light weight and high resistance, by providing an inner layer of a plastics sanitary article with a specific wall thickness, and forming the inner layer and an outer layer as the reaction molded parts. CONSTITUTION: An inner layer has a wall thickness of at least 1 mm, and a non-plastic plastic, for example, a network acrylic resin or an unsaturated polyester is used. This plastic can provide not only the chemical stability but also the stability in size of an inner shell manufactured as a reaction molded part. An outer layer is made of, for example, a plastic foam, and a polyurethane foam resin, for example, is charged to a periphery at a back side of the inner shell. To further improve the mechanical characteristic, at least a half of a volume of the outer layer is formed by a packed body including a hollow body. A material of the outer layer includes the packed body with a high volume ratio, so that the the outer layer is hardly shrinked when it is hardened. Whereby a screw or the other fastening elements can be drawn out with high stability.

EP1016709

Title:
LACTOBACILLUS HELVETICUS BACTERIUM HAVING HIGH CAPABILITY OF PRODUCING TRIPEPTIDE, FERMENTED MILK PRODUCT, AND PROCESS FOR PREPARING THE SAME

Abstract:

There is provided a fermented milk product that contains lactic acid bacteria capable of producing a large amount of lactotripeptide and a large amount of active ingredient having hypotensive activity and anti-stress effect, and that can be taken pleasantly as foods or beverages. Lactic acid bacteria of Lactobacillus helveticus having specific bacteriological properties, the bacteria, when cultured in a medium of animal milk containing 9wt% solid of non-fat milk, producing tripeptides Val-Pro-Pro and Ile-Pro-Pro in an amount of 60 mu g in terms of Val-Pro-Pro per ml medium, and the bacteria exhibiting extracellular proteinase activity of not lower than 400U/OD590. Lactobacillus helveticus CM4 strain (deposited at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, deposition number FERM BP-6060). A fermented milk product obtained by fermenting an animal milk with these lactic acid bacteria.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 016 709 A1

(12) EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:
05.07.2000 Bulletin 2000/27

(21) Application number: **98901520.1**

(22) Date of filing: **05.02.1998**

(51) Int. Cl.[7]: **C12N 1/20**, A23C 9/123
// C12N1:20, C12R1:225

(86) International application number:
PCT/JP98/00481

(87) International publication number:
WO 99/16862 (08.04.1999 Gazette 1999/14)

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
RO SI

(30) Priority: **26.09.1997 JP 27794997**

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(54) LACTOBACILLUS HELVETICUS BACTERIUM HAVING HIGH CAPABILITY OF PRODUCING TRIPEPTIDE, FERMENTED MILK PRODUCT, AND PROCESS FOR PREPARING THE SAME

(57) There is provided a fermented milk product that contains lactic acid bacteria capable of producing a large amount of lactotripeptide and a large amount of active ingredient having hypotensive activity and anti-stress effect, and that can be taken pleasantly as foods or beverages. Lactic acid bacteria of Lactobacillus helveticus having specific bacteriological properties, the bacteria, when cultured in a medium of animal milk containing 9wt% solid of non-fat milk, producing tripeptides Val-Pro-Pro and Ile-Pro-Pro in an amount of 60μg in terms of Val-Pro-Pro per ml medium, and the bacteria exhibiting extracellular proteinase activity of not lower than $400U/OD_{590}$. Lactobacillus helveticus CM4 strain (deposited at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, deposition number FERM BP-6060). A fermented milk product obtained by fermenting an animal milk with these lactic acid bacteria.

EP 1 016 709 A1

Description

Field of Art

[0001] The present invention relates to novel lactic acid bacteria of Lactobacillus helveticus that can produce a particular tripeptide with high efficiency when they are cultured in an animal milk medium and that have high extracellular proteinase activity; a fermented milk product containing the lactic acid bacteria; and a method for producing the product.

Background Art

[0002] Lactobacillus helveticus has been employed for producing fermented milk for a long time as a typical lactic acid bacteria starter for dairy milk products. Lactobacillus helveticus has high proteolytic activity, and particularly, its extracellular proteinase having high activity plays an important role in fermentation of animal milk. That is, the extracellular proteinase digests animal milk proteins to produce various peptide fragments. The produced peptides are further subjected to the action of peptidases to become peptides of lower molecular weight. It is known that a part of peptides produced in a medium due to the action of proteinase enzymes is taken into cells of the lactic acid bacteria and utilized as a nitrogen source. It has also been reported that some of the peptides produced in the medium have an inhibitory activity against angiotensin converting enzyme (ACE) which causes hypertension. (J. Dairy Sci. 78:777-783(1995)).

[0003] As peptides for inhibiting ACE activity and suppressing rise in blood pressure, various effective peptides have been reported, such as those derived from degradation products of milk proteins, soybean proteins or fish meat proteins. For example, Val-Pro-Pro and Ile-Pro-Pro (abbreviated hereinbelow as VPP and IPP, respectively. These peptides are collectively referred to hereinbelow as lactotripeptides) are known as peptides having ACE inhibitory activity present in a Lactobacillus helveticus-fermented milk. These lactotripeptides have been confirmed to have a strong hypotensive effect by experiments using spontaneously hypertensive rat (SHR) (J.Dairy Sci. 78:1253-1257(1995)).

[0004] However, the lactotripeptide-containing fermented milk produced by fermenting animal milk with conventional Lactobacillus helveticus strains can hardly be taken as it is, because it exhibits high acidity due to a large quantity of lactic acid generated as the fermentation progresses. Dilution of the fermented milk results in extreme decrease in the content of the lactotripeptides.

[0005] Thus, it is desired to produce fermented milk with higher content of the lactotripeptides compared to the content of the lactic acid generated in the fermented milk. With an addition of a small amount of such fermented milk to various foods and beverages, products having the function of the lactotripeptides could be prepared easily and provided to consumers in an agreeable form to take. However, none of known lactic acid bacteria strains produce the lactotripeptide with high efficiency.

Disclosure of the Invention

[0006] It is an object of the present invention to provide a novel lactic acid bacteria strain which can produce a large amount of lactotripeptide with high efficiency with respect to the amount of the generated lactic acid.

[0007] It is another object of the present invention to provide a fermented milk product which contains the lactotripeptide having activities such as hypotensive activity and expected to have anti-stress effect, and a lactic acid bacteria strain capable of producing a large amount of this lactotripeptide and which can be taken pleasantly as foods or beverages, and a method for producing the same.

[0008] According to the present invention, there is provided lactic acid bacteria of Lactobacillus helveticus having the following bacteriological properties, said bacteria, when cultured in a medium of animal milk containing 9wt% solid of non-fat milk, producing tripeptides Val-Pro-Pro and Ile-Pro-Pro in an amount of not less than 60μg in terms of Val-Pro-Pro per ml medium, and said bacteria exhibiting extracellular proteinase activity of not lower than 400U/OD_{590}:

(Morphological Properties)

1) Shape of Cell; rod,
2) Motility; none,
3)Spore Formation; none,
4) Gram Stain; positive

(Physiological Properties)

1) Catalase Production; negative,
2) Indole Production; negative,

3) Nitrate Reduction; negative,
4) Aerobic Growth; facultative anaerobic,
5) Formation of DL-lactic acid from glucose by homolactic fermentation without formation of gases
6) Carbohydrate Degradation

glucose; +
lactose; +
mannose; +
fructose; +
galactose; +
sucrose; -
maltose; -
xylose; -
rhamnose; -
cellobiose; -
trehalose; -
melibiose; -
raffinose; -
stachyose; -
mannitol; -
sorbitol; -
esculin; -
salicin; -.

[0009] According to the present invention, there is also provided the lactic acid bacteria of Lactobacillus helveticus wherein said lactic acid bacteria is Lactobacillus helveticus CM4 strain (deposited at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology on August 15, 1997, deposition number FERM BP-6060).

[0010] According to the present invention, there is further provided the lactic acid bacteria of Lactobacillus helveticus having a chromosomal DNA which gives a DNA fragment of 15 to 17 kb when said chromosomal DNA is digested with restriction enzymes PstI and EcoRI.

[0011] According to the present invention, there is also provided a fermented milk product containing a fermented milk comprising the aforementioned lactic acid bacteria, and a tripeptide selected from the group consisting of Val-Pro-Pro, Ile-Pro-Pro and mixtures thereof.

[0012] According to the present invention, there is also provided a method for producing a fermented milk product comprising fermenting a medium containing a food material selected from the group consisting of a peptide, a protein and mixtures thereof including sequence Val-Pro-Pro and Ile-Pro-Pro, with the lactic acid bacteria.

Brief Description of Drawings

[0013]

Fig.1 is a photograph showing the agarose gel electrophoresis pattern of chromosomal DNA fragments of various Lactobacillus helveticus strains in Example 2.

Preferred Embodiments of the Invention

[0014] The lactic acid bacteria of the present invention belong to Lactobacillus helveticus, and characterized in that the lactic acid bacteria produce tripeptides Val-Pro-Pro and Ile-Pro-Pro in an amount of not less than 60μg, and preferably not less than 70μg in terms of Val-Pro-Pro per ml medium when cultured in a medium of animal milk containing 9wt% solid of non-fat milk, and exhibits extracellular proteinase activity of not lower than 400U/OD_{590}, and preferably not lower than 430U/OD_{590}. The defined lactotripeptide productivity is an index to distinguish the present lactic acid bacteria from conventional lactic acid bacteria of Lactobacillus helveticus. For example, by this index is defined a property of the present lactic acid bacteria to produce, when cultured in animal milk containing 9wt% solid of non-fat milk, the lactotripeptides in an amount of not less than 60μg in terms of VPP per ml medium, which could not have been realized with the conventional lactic acid bacteria. Usually, the lower the content of the solid of non-fat milk in the medium for culturing, the smaller the amount of the lactotripeptides to be produced. The higher the content of the solid of non-fat milk, the larger the amount of the lactotripeptide.

[0015] The productivity of the lactotripeptides as the index is measured by the steps of inoculating with the lactic acid bacteria animal milk, such as cow's milk, goat's milk, horse's milk, and skim milks thereof, containing 9wt% solid of non-fat milk, culturing the bacteria at 37°C for 24 hours to prepare fermented milk, centrifuging 1ml of the fermented milk at 15,000rpm for 10 minutes, subjecting the supernatant to measurement for the amounts of VPP and IPP, and converting the amounts into the VPP amount. Converted amount of the lactotripeptide in terms of VPP is calculated by the following equation since ACE inhibitory activity of IPP per unit weight is 1.7 times that of VPP;

Converted amount of lactotripeptide (μg in terms of VPP per ml)
= amount of IPP (μg/ml) x 1.7 + amount of VPP (μg/ml)

[0016] The maximum lactotripeptide productivity is not particularly limited, but can be achieved when all of Val-Pro-Pro and Ile-Pro-Pro included as the sequences in the protein of the medium are taken out as the tripeptides by digestion.

[0017] The extracellular proteinase activity is measured in accordance with the method of Yamamoto et al. (Yamamoto, N. et al. J.Biochem.(1993)114, 740) based on the method of Twining et al. (Twining,S. Anal.Biochem. 143 3410 (1984)), and expressed by defining the amount of enzyme exhibiting 1% fluorescent intensity to be $1U/OD_{590}$. The upper limit of the extracellular proteinase activity is not limited either, but is usually $800U/OD_{590}$.

[0018] The present lactic acid bacteria can produce a large amount of the lactotripeptide with respect to the amount of the lactic acid generated during fermentation. Thus, fermentation using the present lactic acid bacteria results in a fermented milk containing a larger amount of the lactotripeptide compared to a fermented milk containing the similar amount of lactic acid prepared with conventional lactic acid bacteria. The lactic acid due to such fermentation is DL-lactic acid. The amount of the lactotripeptide produced by fermentation with the present lactic acid bacteria is preferably not less than 30μg in terms of VPP per 1ml of the resulting fermented milk containing 0.01g/ml of DL-lactic acid generated during the fermentation. The upper limit of the amount of the lactotripeptide is not particularly limited, but it is possible for the bacteria to produce up to about 50μg in terms of VPP per 1ml of the fermented milk containing 0.01g of DL-lactic acid. The amount of DL-lactic acid is roughly proportional to the amount of the lactotripeptide. Therefore, for example, when 0.02g of DL-lactic acid is produced in 1ml of the fermented milk, the amount of the lactotripeptide production is preferably not less than 60μg in terms of VPP. On the contrary, by the fermentation with the conventional lactic acid bacteria, the amount of the lactotripeptide is, at most, less than 30μg in terms of VPP per 0.01g of DL-lactic acid in 1ml of fermented milk.

[0019] As an example of the present lactic acid bacteria, Lactobacillus helveticus CM4 strain is deposited as FERM BP-6060 at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (deposited on August 15, 1997). Lactobacillus helveticus CM4 strain has the following bacteriological properties:

1. Morphological Properties

1) Shape of Cell; rod,
2) Motility; none,
3) Spore Formation; none,
4) Gram Stain; positive

2. Physiological Properties

1) Catalase Production; negative,
2) Indole Production; negative,
3) Nitrate Reduction; negative,
4) Aerobic Growth; facultative anaerobic,
5) Formation of DL-lactic acid from glucose by homolactic fermentation without formation of gases
6) Carbohydrate Degradation

glucose; +
lactose; +
mannose; +
fructose; +
galactose; +
sucrose; -
maltose; -

xylose; -
rhamnose; -
cellobiose; -
trehalose; -
melibiose; -
raffinose; -
stachyose; -
mannitol; -
sorbitol; -
esculin; -
salicin; -

The aforementioned bacteriological properties of CM4 strain are identical with publicly known Lactobacillus helveticus NCDO-099 strain when examined by the method of Mitsuoka et al. (Rinshoukensa 18, 1163(1974)). However, as to the following properties, which are not described in Mitsuoka et al., CM4 is clearly distinguished from NCDO-099.

7) Extracellular proteinase activity; not less than 400U/OD_{590}

8) Lactotripeptide productivity; production of two sorts of tripeptides (VPP and IPP) in an amount of 60μg or more in terms of VPP per ml fermented liquid when cultured in a medium containing 9wt% skim milk at 37° C for 24 hours

[0020] The lactotripeptide productivity in 8) is measured using skim milk as solid of non-fat milk.

[0021] The lactic acid bacteria strain of the present invention may be obtained by the following screening and measurement of extracellular proteinase activity.

(1) Primary screening

(selection of strain by measurement of high ACE inhibitory activity in the fermented milk)

[0022] The strains to be screened are cultured in a 9wt% skim milk medium at 37°C for 24 hours. After finishing the culturing, the number of the lactic acid bacteria, acidity of the lactic acid, and ACE inhibitory activity are measured. Strains producing $1x10^8$ cells/ml or more of lactic acid bacteria, and exhibiting acidity of the lactic acid of 1.6wt% or more and ACE inhibitory activity of 40 unit/ml or more are selected. ACE inhibitory activity is measured by Cushman and Cheung's method (Cushman,D.W. and Cheung,H.S. Pharmacol., 20 1637(1971)).

(2) secondary screening

(selection of strain having high lactotripeptide productivity)

[0023] The cultured liquids of the strains selected by the primary screening are centrifuged at 15,000rpm for 10 minutes, and the supernatants thereof are subjected to HPLC for quantifying the lactotripeptide. Strains which produced not less than 50μg in terms of VPP per ml are selected.

(3) Measurement of extracellular proteinase activity

[0024] Each of the strains selected by the secondary screening is cultured in a 9wt% skim milk medium while pH thereof is maintained at 6. Sample is taken in the middle of logarithmic growth phase, and admixed with 1 wt% of sodium citrate, and centrifuged at 5,000rpm for 10 minutes to harvest cells. The harvested cells were washed with 50mM β-glycerophosphoric acid, and suspended in 50mM Tris-HCl buffer (pH7.8) to adjust turbidity (OD_{590}) to 1. Proteinase activity on the cell surface is then measured. It will be confirmed that the result is correlative with lactotripeptide productivity of strains measured in the secondary screening.

[0025] The lactic acid bacteria strain of Lactobacillus helveticus selected by the above method can be identified and distinguished from other lactic acid bacteria strains by, e.g., the aforementioned lactotripeptide productivity and extracellular proteinase activity.

[0026] The lactic acid bacteria of the present invention preferably has, in addition to the aforementioned lactotripeptide productivity and extracellular proteinase activity, chromosomal DNA which gives a DNA fragment of 15 to 17 kb when the chromosomal DNA is digested with restriction enzymes PstI and EcoRI. Therefore, the lactic acid bacteria of

the present invention can clearly be distinguished from other strains of the same species by examining whether the strain has the chromosomal DNA which gives such a DNA fragment.

[0027] The existence of the DNA fragment of 15 to 17 kb may be confirmed by extracting the chromosomal DNA of the lactic acid bacteria in accordance with the method of Leenhouts et al. (Leenhouts, K.(1990) Appl.Environ.Microbiol. 56:2726), digesting the chromosomal DNA with EcoRI and PstI, performing 0.8% agarose gel electrophoresis of the digested fragments, and analyzing the resulting electrophoresis pattern. Upon electrophoresis, the existence of the DNA fragment is clearly confirmed by subjecting λ phage DNA digested with a restriction enzyme Hind III to parallel electrophoresis as a size marker.

[0028] The fermented milk product of the present invention contains, as a requisite component, fermented milk containing the lactic acid bacteria and the tripeptide selected from the group consisting of VPP, IPP and mixtures thereof. That is, the fermented milk product of the present invention contains fermented milk containing the lactotripeptide and the lactic acid bacteria, and prepared by fermentation of a medium containing a food material composed of peptides and/or proteins including the sequence VPP and/or IPP with the lactic acid bacteria of the present invention. Thus, the contents of the lactic acid bacteria and the tripeptide may be suitably selected depending on the sort of the fermented milk product to be prepared. The present fermented milk product may contain the obtained fermented product itself, a diluted fermented product, or a purified fermented product.

[0029] The fermented milk product of the present invention contains DL-lactic acid generated during the fermentation. The fermented milk product of the present invention preferably contains the lactotripeptide in an amount of 30 to 50 μg in terms of VPP with respect to 0.01g of the DL-lactic acid. The amount of the DL-lactic acid is roughly proportional to the amount of the lactotripeptide. Thus, if the fermented milk product contains a concentrated fermented milk and contains, e.g., 0.02g of the DL-lactic acid, the amount of the lactotripeptide is preferably in a range of 60 to 100μg in terms of VPP. If the fermented milk product contains a diluted fermented milk and contains, e.g., 0.005g of the DL-lactic acid, the amount of the lactotripeptide is preferably 15 to 25μg in terms of VPP. Although the fermented milk product of the present invention may contain L-lactic acid, which is a food additive for adjusting acidity, this L-lactic acid is to be distinguished from the DL-lactic acid generated during the fermentation.

[0030] The lactic acid bacteria in the fermented milk product of the present invention may be either sterilized after fermentation, or kept alive without sterilization.

[0031] The fermented milk product of the present invention may be yogurt, milk-containing acidified beverages, cheese, processed foods containing fermented sour milk, and healthy foods containing fermented sour milk. Thus, the fermented milk product of the present invention may contain, in addition to the fermented milk as the requisite component, various materials which are usually added for producing such a variety of products. The fermented milk product of the present invention may be in the form of solid such as powders, granules and tablets, or of fluid such as paste, gel and liquid.

[0032] The method for producing the fermented milk product of the present invention includes fermenting with the lactic acid bacteria a medium containing a food material selected from the group consisting of a peptide, a protein and mixtures thereof including Val-Pro-Pro and/or Ile-Pro-Pro as a part of its sequence.

[0033] The food material in the medium may be of any kind as long as it contains peptides and/or proteins including, as a part of their sequence, VPP and/or IPP. For example the food material may be animal milk, milk casein, corn, corn protein, wheat, wheat protein, soybean, soybean milk, de-fat soybean, soybean protein, or mixtures thereof. Particularly, it is preferable to employ a food material containing animal milk such as cow's milk, goat's milk, horse's milk, or skim milks of these. The content of the solid of non-fat milk in the animal milk is not particularly limited, but is usually 5 to 20wt%.

[0034] There is no particular limitation on the amount of the lactic acid bacteria with which the medium is inoculated. The inoculation amount is usually about 10^5 to 10^7 cells of the lactic acid bacteria per 1g of the aforementioned specific food material in the medium.

[0035] The fermentation may be performed at 25 to 50° C and preferably 30 to 45° C, for 6 to 30 hours and preferably 10 to 24 hours, in the pH range of preferably 3.0 to 4.0, and more preferably 3.0 to 3.5.

[0036] The fermentation is preferably performed such that the amount of the lactotripeptide is not less than 60μg in terms of VPP per ml of the resulting fermented milk. Specifically, when cow's milk containing 9wt% solid of non-fat milk is employed as a medium, fermentation at 25 to 40° C for 12 to 48 hours results in a fermented milk containing the lactotripeptide in an amount of not less than 70μg in terms of VPP per ml. The content of the solid of non-fat milk in the medium is roughly proportional to the lactotripeptide to be produced. For example, if the food material contains 5wt% solid of non-fat milk, the fermentation in accordance with the aforementioned conditions would result in production of the lactotripeptide in an amount of about 33.3 μg in terms of VPP per ml.

[0037] The fermented milk obtained by the aforementioned fermentation may be admixed with the product as it is. If necessary, the fermented milk may be subjected to dilution or purification before mixing. The fermented milk may be cooled and stored at 5°C, and then admixed with other components to prepare a product such as a chilled product. Alternatively, the fermented milk may be subjected to heat sterilization treatment, and, if necessary, powdered by spray

drying to prepare a product for distributing at an ordinary temperature.

[0038] Since the fermented milk product of the present invention contains the fermented milk obtained by fermentation with the lactic acid bacteria, it can be used to prepare easily products having high content of the lactotripeptide with respect to the content of the lactic acid, in an agreeable form to take. The fermented milk product is expected to exhibit hypotensive effect and anti-stress effect of the lactotripeptide when taken by human being.

[0039] Since the lactic acid bacteria of the present invention can produce a large amount of the lactotripeptide by culturing them in the specific food material, the bacteria are useful in producing a variety of fermented milk products, functional foods, healthy foods, foods for specified health use, foods for specified use for elder people, and the materials thereof, having hypotensive effect and stress-relieving effect of the lactotripeptide.

Examples of the Invention

[0040] The present invention will be explained more in detail hereinbelow referring to the Examples, but the present invention is not limited thereto.

[0041] Among the Lactobacillus helveticus strains used in the Examples, CM4 strain is deposited at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology and has been accorded accession number FERM BP-6060. ATCC15009, NCDO-099, JCM1006, ATCC10797, JCM1062, JCM1103, JCM1120 and JCM1004 are publicly available strains. Strains other than the above strains used in the Examples are selected from strain collection of the applicant.

Example 1

(Selection of strains giving fermented milk having high ACE inhibitory activity)

[0042] 36 strains of Lactobacillus helveticus isolated from various dairy products were screened. ACE inhibitory activity of milk fermented with each of the strains was measured by the following procedure. Each of the Lactobacillus helveticus strains was cultured in a 9wt% solid of non-fat milk medium at 37° C for 24 hours. The cultured medium was added to a fresh medium of the same type in such an amount that the new medium contains 3wt% of the cultured medium. Fermentation was further performed at 37° C for 24 hours. After finishing the fermentation, acidity of lactic acid (wt%), the amount of the peptide in the whey (mg/ml), the number of cells and ACE inhibitory activity (U/ml) were measured. The results are shown in Table 1. 7 strains out of 36 strains had very weak fermentation ability. 15 strains produced fermented milk with the acidity of the lactic acid generated of not less than 1.6 wt%. Out of the 15 strains, 8 strains having ACE inhibitory activity of not less than 40U/ml whey in its fermented milk were selected.

(Measurement of ACE inhibitory activity of fermented milk)

[0043] ACE inhibitory activity was measured in accordance with Cushman and Cheung's method (Cushman,D.W. and Cheung,H.S. Pharmacol., 20 1637(1971)). That is, each of the fermented milk was centrifuged at 15,000rpm for 5 minutes to obtain the supernatant (whey). The whey was suitably diluted for measurement. 80μl of the diluted whey was put in a tube, admixed with 0.2 ml of 0.1M boric acid buffer (containing 0.3M NaCl, pH7.3) containing 5mM hippuryl histidine leucine (Hip-His-Leu, manufactured by SIGMA CHEMICALS CO.) as a substrate, and further admixed with 20μl of enzyme solution (0.1U/ml, manufactured by SIGMA CHEMICALS CO.). The resulting mixture was reacted at 37°C for 30 minutes, and then admixed with 250μl of 1N hydrochloric acid for terminating the reaction. Subsequently, the mixture was admixed with 1.7ml of ethyl acetate, stirred for 20 seconds, and then centrifuged at 3,000rpm for 10 minutes to recover 1.4ml of ethyl acetate phase (upper phase). The upper phase was heated at 120°C for 40 minutes to remove the solvent, admixed with 1ml of distilled water, and stirred for about 20 seconds. The hippurylic acid extracted was measured for absorbance at 228nm. The enzyme activity in unit was calculated by the following equation with the amount which gives 50% inhibition of the ACE activity being defined as one unit.

$$\text{Amount of the enzyme (unit)} = ((A-B)/(A-C)) \times 100 \times 1/50$$

A: Absorbance at 228nm without sample
B: Absorbance at 228nm with sample
C: Absorbance at 228 without enzyme nor sample

(Quantitative analysis of amount of peptides in the fermented milk)

[0044] Quantitative analysis of the peptides was performed in accordance with OPA method (Charch,F.C. et al.

J.Dairy Sci. 66 1219(1883). As a standard substance for generating a calibration curve, casein digested with trypsin was employed.

Table 1

Strains	Acidity (wt%)	Amount of Peptides (mg/ml)	Number of Cells ($x10^8$cells /ml)	ACE Inhibitory activity(U /ml)
strain 1	-	-	-	-
strain 2	-	-	-	-
strain 3	-	-	-	-
strain 4	-	-	-	-
strain 5	-	-	-	-
strain 6	-	-	-	-
strain 7	-	-	-	-
strain 8	0.498	1.59	0.29	26.4
strain 9	2.022	1.99	9.53	34.5
strain 10	1.709	2.10	8.53	24.5
strain 11	0.615	1.76	1.28	29.1
strain 12	0.411	1.35	0.38	17.6
strain 13	0.917	1.57	3.63	19.9
strain 14	1.026	1.71	5.78	9.4
strain 15	0.517	1.59	0.56	26.9
strain 16	1.532	4.69	5.97	102.5
strain 17	2.101	2.01	6.09	98.9
strain 18	1.783	1.94	5.38	21.9
strain 19	1.955	1.69	5.31	100.6
strain 20	2.095	1.74	7.16	61.4
strain 21	1.963	2.03	6.05	125.3
strain 22	1.798	2.85	6.19	54.2
strain 23	1.604	2.32	6.81	36.6
strain 24	1.932	1.77	7.97	47.7
strain 25	1.885	1.51	4.91	18.3
strain 26	1.862	1.46	5.69	26.2
strain 27	1.063	3.01	2.78	76.9
strain 28	0.457	1.98	0.50	52.4
strain 29	0.516	2.55	1.13	92.6
JCM1006	1.872	2.35	6.97	48.5
JCM1062	1.109	2.60	8.50	78.4
JCM1103	1.244	1.36	3.69	31.0
ATCC10797	1.359	2.11	8.56	13.8
ATCC15009	1.454	1.81	5.16	16.6
NCDO-099	1.769	2.76	6.59	25.5

Table 1 (continued)

Strains	Acidity (wt%)	Amount of Peptides (mg/ml)	Number of Cells ($x10^8$cells /ml)	ACE Inhibitory activity(U /ml)
CM4	1.635	3.12	4.44	130.0

(Selection of strains having high lactotripeptide productivity)

[0045] Subsequently, the 8 strains which gave fermented milk having high ACE inhibitory activity as selected above were measured for VPP and IPP in their fermented milk.

[0046] 1ml of the fermented milk was centrifuged at 15,000rpm for 10 minutes. The supernatant thereof, i.e. whey, was collected. 0.3ml of the whey was subjected to Sep-Pak Cartridge (manufactured by WATERS INC.) adsorption, washed with distilled water, and then eluted with 5ml of methanol. The eluate was dried under centrifugation and reduced pressure. The dried product was dissolved in 0.3ml of a 0.05% aqueous solution of trifluoroacetic acid, and subjected to HPLC (high performance liquid chromatography) analysis under the following conditions. The results are shown in Table 2.

Apparatus Employed:

Hitachi L4000UV detector (at 215nm)
L6200 intelligent pump
L5030 column oven (35°C)

Condition of Elution: Flow rate 0.5ml/min.
Eluent: Aqueous solution containing 0.3M NaCl and 0.05% trifluoroacetic acid
Column: Asahipak GS320 (Φ3.9x600mm)

[0047] Since the ACE inhibitory activity of IPP per unit weight is 1.7 times that of VPP, the amount of the lactotripeptides in terms of VPP was calculated from the measured amounts of IPP and VPP in accordance with the following equation. The results are shown in Table 2.

Converted amount of lactotripeptide (μg in terms of VPP per ml)=Amount of IPP (μg/ml)x1.7 + Amount of VPP (μg/ml)

Table 2

Strains	Amount of Peptide (μg/ml whey)			Acidity (wt%)
	VPP	IPP	Amount of lactotripeptide in terms of VPP	
Strain 17	15.2	11.1	34.0	1.5
Strain 19	11.2	7.3	23.7	1.4
Strain 20	13.0	8.1	26.8	1.6
Strain 21	16.6	11.4	36.0	1.6
Strain 22	15.8	12.1	36.3	1.5
Strain 24	12.6	8.7	27.4	1.6
JCM1006	12.9	9.3	28.6	1.3
CM4	38.5	23.5	78.5	1.9

[0048] CM4 fermented milk had the largest amount of the lactotripeptide in terms of VPP, that is, 78.5μg/ml whey. Other seven strains gave the average amount of 34.2μg/ml whey.

(Measurement of extracellular proteinase)

[0049] Extracellular proteinase activity was measured of 16 strains which gave relatively good results in fermentability shown in Table 1. Measurement was performed in accordance with the method of Yamamoto et al. (Yamamoto, N. et al. J.Biochem. (1993) 114, 740) based on the method of Twining et al. (Twining,S. Anal.Biochem. 143 3410 (1984)). That is, each strain was cultured in 9wt% skim milk medium while pH thereof was maintained at 6.0. Sample was taken in the middle of logarithmic growth phase, and admixed with sodium citrate so that the final concentration was 1wt%, for clarifying the milk medium. The mixture was centrifuged at 5,000rpm for 10 minutes to collect cells. The cells were washed with 50mM β-glycerophosphoric acid, and suspended in 50mM Tris-HCl buffer (pH7.8) to adjust turbidity (OD_{590}, i.e. measured by absorbance at 590nm) to 1. 30μl of the suspension was admixed with 20μl of 0.4% fluorescein-casein (manufactured by SIGMA CHEMICALS CO.), and incubated at 42°C for 1 hour. The mixture was further admixed with 120μl of 5% trichloroacetic acid, allowed to stand for 20 minutes, and centrifuged at 15,000rpm for 10 minutes. 60μl of the supernatant was added to 3ml of 500mM tris-HCl buffer (pH 8.3), and the fluorescent intensity thereof was measured by detecting the fluorescence of 525nm produced at an excitation wavelength of 490nm. Extracellular proteinase activity in unit was calculated with the amount of the enzyme which exhibits 1% fluorescent intensity under the above conditions being defined as one unit. The results are shown in Table 3.

Table 3

Strains	U/OD_{590}
strain 17	136.7
strain 18	102.8
strain 19	103.2
strain 20	89.9
strain 21	80.1
strain 22	243.3
strain 23	116.6
strain 24	116.6
strain 25	192.6
strain 26	108.4
JCM1006	185.7
JCM1062	96.5
JCM1103	176.3
ATCC15009	168.1
ATCC10797	106.5
NCDO-099	229.7
CM4	452.6

[0050] The activity of Lactobacillus helveticus CM4 was the highest, that is, $450U/OD_{590}$. Average activity for other 16 strains was $141U/OD_{590}$, which is about one third of that of CM4 strain.

Example 2

[0051] From 11 strains out of 36 Lactobacillus helveticus strains selected in Example 1, chromosomal DNA was extracted in accordance with the method of Leenhouts et al. (Leenhouts, K. (1990) Appl.Environ.Microbiol. 56:2726), digested with several restriction enzymes, and subjected to 0.8% agarose gel electrophoresis to analyze the electrophoresis pattern.

[0052] As a result, a characteristic DNA fragment was observed among DNA fragments of chromosome of CM4 strain digested with EcoRI and PstI (shown by arrow 1 in Fig.1). Such a fragment was not observed in the fragments of

chromosomes from other strains than CM4, and shorter fragments than the characteristic fragment of CM4 were observed in most of other strains (shown by arrow 2 in Fig.1). The molecular weight of the characteristic fragment was measured by comparative electrophoresis of the Hind III digestion products of λ phage DNA as size markers (23.1kb, 9.4kb, 6.6kb, 4.4kb, 2.3kb and 2.0kb, in the order of increasing mobility), and was found to be about 16kb. Thus, it was confirmed that CM4 strain has a chromosomal DNA which gives the DNA fragment having molecular weight of about 16kb, by digestion with EcoRI and PstI. It was also confirmed that other strains than CM4 have chromosomal DNA which give a common DNA fragment having molecular weight of about 13kb.

Example 3

[0053] A fermented milk was produced with Lactobacillus helveticus CM4 strain selected in Example 1. CM4 strain was cultured in 100g of 9wt% skim milk at 37°C for 12 hours. Subsequently, 3kg of fresh medium was inoculated with the cultured skim milk, and cultured at 37°C for 12 hours. After finishing the fermentation, all of the fermented milk (number of cells of CM4 strain; $6.3x10^8$ cells/ml) was used as a starter for fermentation of 100kg of 9wt% skim milk at 32°C for 20 hours. After finishing the fermentation, 74.8μg/ml of the lactotripeptide was contained in the fermented milk. The content of the lactic acid was 1.9wt%.

[0054] 43kg of the obtained fermented milk was admixed with 4kg of granulated sugar, 3kg of water and 0.15kg of high methoxypectin, and homogenized to obtain 50kg of yogurt drink. The yogurt beverage had a preferable mild taste, pH of 3.6 and $4.6x10^8$ cells/g of live CM4 cells.

Example 4

[0055] 26.5kg of the fermented milk obtained in Example 3 was admixed with 45.0kg of granulated sugar, 4.7kg of high maltose syrup and 13.8kg of water. 10kg of 3wt% high methoxypectin solution was added to the mixture under stirring. The resulting mixture was homogenized using a laboratory homogenizer (manufactured by ATV GAULIN, INC., Model 15M-8BA) under a treatment pressure of 150kg/cm^2 and at a treatment flow rate of 2500ml/min. The homogenized liquid was admixed with a vanilla flavor and sterilized by heating up to 85°C. The fermented milk thus sterilized was charged in a 200ml glass bottle while hot. The content of the lactotripeptide in the sterilized fermented milk product was measured. It was found out that the content of the lactotripeptide corresponded to that in the fermented milk before sterilization. It was also found out that the content of the lactic acid was 0.5wt%.

Claims

1. Lactic acid bacteria of Lactobacillus helveticus having the following bacteriological properties, said bacteria, when cultured in a medium of animal milk containing 9wt% solid of non-fat milk, producing tripeptides Val-Pro-Pro and Ile-Pro-Pro in an amount of not less than 60μg in terms of Val-Pro-Pro per ml medium, and said bacteria exhibiting extracellular proteinase activity of not less than 400U/OD_{590}:

(Morphological Properties)

1) Shape of Cell; rod,
2) Motility; none,
3) Spore Formation; none,
4) Gram Stain; positive

(Physiological Properties)

1) Catalase Production; negative,
2) Indole Production; negative,
3) Nitrate Reduction; negative,
4) Aerobic Growth; facultative anaerobic,
5) Formation of DL-lactic acid from glucose by homolactic fermentation without formation of gases
6) Carbohydrate Degradation

glucose; +
lactose; +
mannose; +
fructose; +

galactose; +
sucrose; -
maltose; -
xylose; -
rhamnose; -
cellobiose; -
trehalose; -
melibiose; -
raffinose; -
stachyose; -
mannitol; -
sorbitol; -
esculin; -
salicin, -.

2. The lactic acid bacteria of Lactobacillus helveticus of claim 1 wherein said lactic acid bacteria is Lactobacillus helveticus CM4 strain (deposition number at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology; FERM BP-6060).

3. The lactic acid bacteria of Lactobacillus helveticus of claim 1 wherein said bacteria have a chromosomal DNA which gives a DNA fragment of 15 to 17 kb when said chromosomal DNA is digested with restriction enzymes PstI and EcoRI.

4. A fermented milk product comprising the lactic acid bacteria of claim 1, and a tripeptide selected from the group consisting of Val-Pro-Pro, Ile-Pro-Pro and mixtures thereof.

5. The fermented milk product of claim 4 further comprising DL-lactic acid, wherein said product contains 30 to 50μg of said tripeptide in terms of Val-Pro-Pro per 0.01g of said DL-lactic acid.

6. The fermented milk product of claim 4 wherein the lactic acid bacteria of claim 1 are alive.

7. The fermented milk product of claim 4 wherein said fermented milk product is selected from the group consisting of yogurt, acidic milk beverages, cheese, processed foods containing fermented sour milk, and healthy foods containing fermented sour milk.

8. A method for producing the fermented milk product of claim 4 comprising fermenting a medium containing a food material selected from the group consisting of a peptide, a protein and mixtures thereof including sequence Val-Pro-Pro and Ile-Pro-Pro, with the lactic acid bacteria of claim 1.

9. The method of claim 8 wherein said food material is selected from the group consisting of animal milk, milk casein, corn; corn protein, wheat, wheat protein, soybean, soybean milk, de-fat soybean, soybean protein, and mixtures thereof.

10. The method of claim 8 wherein the fermentation is performed at 25 to 50° C for 6 to 60 hours.

11. The method of claim 8 wherein the fermentation is performed under such conditions that the amount of tripeptides Val-Pro-Pro and Ile-Pro-Pro produced in resulting fermented milk is 60μg in terms of Val-Pro-Pro per ml.

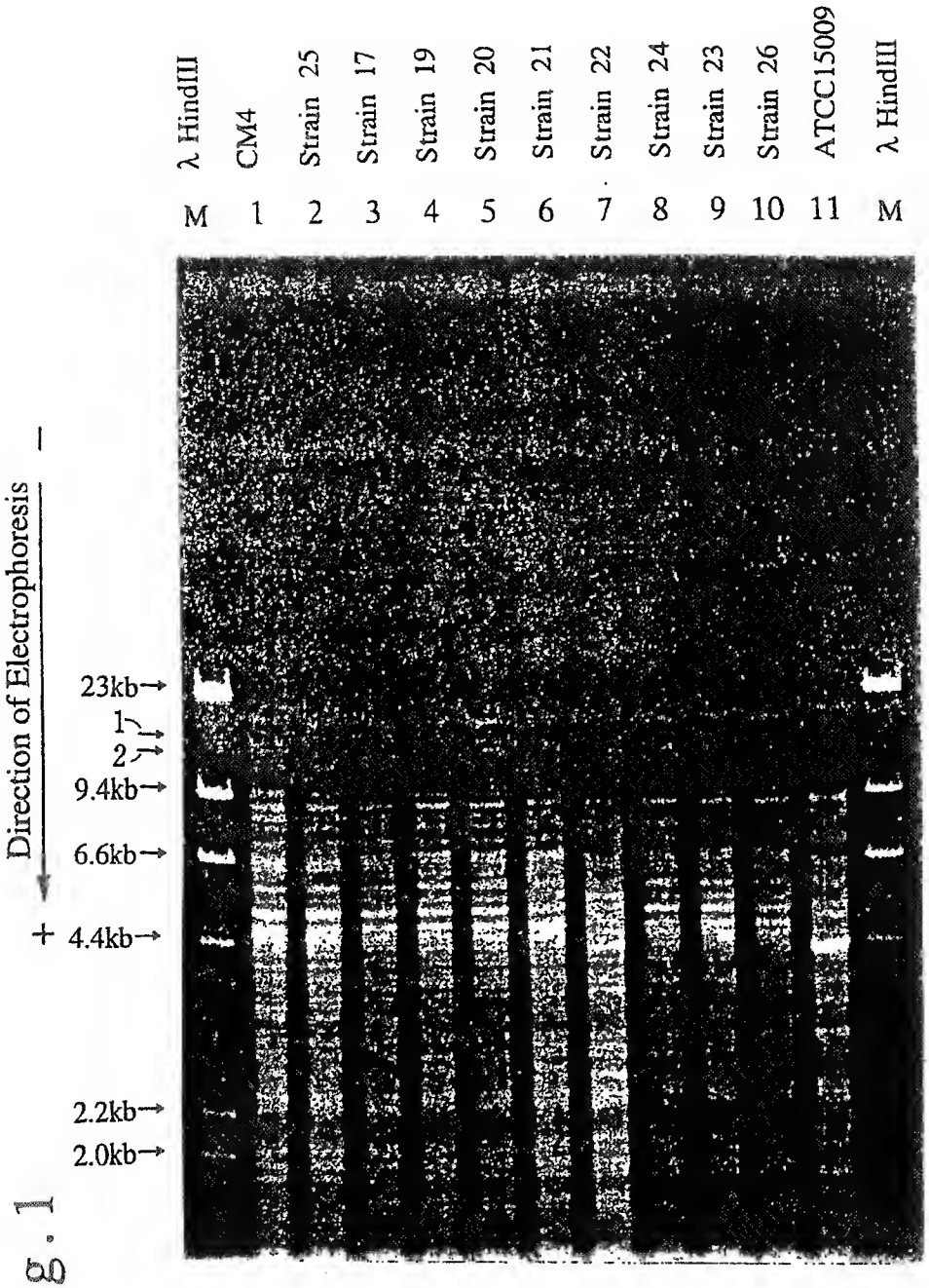

Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/00481

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] C12N1/20, A23C9/123 // (C12N1/20, C12R1:225)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] C12N1/20, A23C9/123

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA (STN), REGISTRY (STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 6-197786, A (The Calpis Food Industry Co., Ltd.), July 19, 1994 (19. 07. 94) (Family: none)	1-11
A	JP, 6-40944, A (The Calpis Food Industry Co., Ltd.), February 15, 1994 (15. 02. 94) & EP, 583074, A & US, 5449661, A	1-11
A	YASUNORI NAKAMURA ; NAOYUKI YAMAMOTO ; KUMI SAKAI ; AKIRA OKUBO ; SUNAO YAMAZAKI ; YOSHIAKI TAKANO, Purification and Characterization of Angiotensin I-Converting Enzime Inhibitors from Sour Milk, Journal of Dairy Science, 1995, Vol. 78, No. 4, p.777-783	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
February 24, 1998 (24. 02. 98)	March 3, 1998 (03. 03. 98)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

EP0966969

Title:
ANTI-STRESS DRUGS AND FUNCTIONAL FOODS HAVING ANTI-STRESS EFFECTS

Abstract:

Anti-stress drugs which can be continuously administered without any problem in safety and relieve or prevent mental and physical symptoms caused by stresses and functional foods having anti-stress effects. The drugs contain as the active ingredients acid milks prepared by fermenting animal milks by using lactic acid bacteria belonging to the genus Lactobacillus , which the functional foods contain these drugs and have anti-stress effects.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 966 969 A1

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 158(3) EPC

(43) Date of publication:
29.12.1999 Bulletin 1999/52

(21) Application number: **97934703.6**

(22) Date of filing: **06.08.1997**

(51) Int. Cl.[6]: **A61K 35/20**, A23L 1/30

(86) International application number:
PCT/JP97/02728

(87) International publication number:
WO 98/05343 (12.02.1998 Gazette 1998/06)

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
RO SI

(30) Priority: **07.08.1996 JP 20850496**

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(54) ANTI-STRESS DRUGS AND FUNCTIONAL FOODS HAVING ANTI-STRESS EFFECTS

(57) Anti-stress drugs which can be continuously administered without any problem in safety and relieve or prevent mental and physical symptoms caused by stresses and functional foods having anti-stress effects. The drugs contain as the active ingredients acid milks prepared by fermenting animal milks by using lactic acid bacteria belonging to the genus *Lactobacillus* , which the functional foods contain these drugs and have anti-stress effects.

EP 0 966 969 A1

Description

[0001] This invention relates to an anti-stress agent having effects of preventing and mitigating mental and physical symptoms caused by stress, and functional food containing the anti-stress agent which is prepared by adding the anti-stress agent to yogurt, milk-containing acidified beverages, cheese, various processed foods, healthy foods, powdered foods, or granulated foods to give an anti-stress effect thereto.

[0002] In the modern society, people undergo various kinds of stress caused by highly advanced and complicated scientific technology, or drastically changing social circumstances. Particularly, in the internationalized society, complex human relationships are formed, causing mental stress. It has been reported that a variety of symptoms are caused by mental stress.

[0003] It is recognized that mental stress has a great influence on circulatory system. However, the scientific concept and definition of stress have not yet been well established, so that means of evaluation of stress still have many problems, combined with methodological difficulties. However, in the recent years, studies of stress have been made from the medical point of view.

[0004] For example, it is reported that when one undergoes stress, angiotensins II and/or vasopressin increase, and intracorporeal sodium due to sodium reabsorbancy becomes excess, which causes rise in blood pressure (Osamu Mobara et al.: Taisha, 28 , 2, 323, 1991). However, suffering stress not only causes rise in blood pressure, but also influences various factors, such that it is believed to cause stomach ulcer, ischemic cardiac diseases, cerebrovascular diseases, hypertension, hyperlipemia, or the like. Therefore, though the investigation of the relationship between stress and hypertension is of importance, it is not believed that mere lowering of blood pressure will bring about the anti-stress effect.

[0005] As an agent for preventing and mitigating mental and physical symptoms caused by stress, chemically synthesized medicaments such as a tranquilizer, an antianxiety agent, and sleeping pills are presently used. However, these medicaments have habituation and side effect problems, so that it is not preferable to use them daily for the purpose of preventing mental and physical symptoms caused by stress. Accordingly, foods having the anti-stress effect are desired and are under development, which can be taken repeatedly and daily without any problems with safety, and which can mitigate and prevent mental and physical symptoms caused by stress. For example, an anti-stress agent containing as an effective ingredient L-theanine contained in tea leaves, is proposed in Japanese Laid-open Patent Application No. 6-100442, which can be mixed in tasty beverages (nutrient supplementary drinks) for daily uptake. Further, a stress relieving effect of fragrance is also reported (FRAGRANCE JOURNAL:1991-11, 44). However, there has not been reported that lactic acid bacteria-fermented milk has the effect of mitigating and preventing mental and physical symptoms caused by stress.

[0006] It is therefore an object of the present invention to provide an anti-stress agent which can fulfill the social demand as mentioned above, which can be taken repeatedly and daily without any problems with safety, and which can mitigate and prevent mental and physical symptoms caused by stress; and its use.

[0007] It is another object of the present invention to provide functional food having an anti-stress effect which can fulfill the social demand as mentioned above, and which can be taken as food repeatedly and daily without any problems with safety; and its use.

[0008] The present inventors have made intensive studies to find a substance which can fulfill the social demand as mentioned above, which can be used in food, and which can be taken repeatedly without any problems with safety. As a result, they have noticed that fermented sour milk has a superior anti-stress effect, and completed the present invention.

[0009] According to the present invention, there is provided an anti-stress agent containing as effective agent at least one fermented sour milk.

[0010] According to the present invention, there is also provided a functional food having anti-stress effect containing the anti-stress agent.

[0011] According to the present invention, there is further provided use of the anti-stress agent or the functional food for the manufacture of a drug for the treatment of stress.

[0012] According to the present invention, there is also provided a method of treatment of human or animal stress comprising oral administration of the anti-stress agent or the functional food.

Fig. 1 is a graph showing the fluctuation in the diastolic blood pressure during the testing period of the mental arithmetic test in Experiment 1 in Example 1. The ordinate of the graph expresses the diastolic blood pressure in mmHg, while the abscissa expresses the time in minute.

Fig. 2 is a graph showing the change in Profile of Mood State (POMS) after the termination of the mental arithmetic test in Experiment 1 in Example 1. The ordinate of the graph expresses the change in POMS score in percent (%).

Fig. 3 is a graph showing the fluctuation in the diastolic blood pressure during the testing period of the mental arithmetic test when the panels were given fermented sour milk beverage for one week in Example 1. The ordinate of the graph expresses the diastolic blood pressure in mmHg, while the abscissa expresses the time in minute.

Fig. 4 is a graph showing the fluctuation in the heart rate during the testing period of the mental arithmetic test when the panels were give fermented sour milk beverage for one week in Example 1. The ordi-

nate of the graph expresses the heart rate per minute in beat/min., while the abscissa expresses the time in minute.

[0013] The anti-stress agent of the present invention contains at least one fermented sour milk as effective agent having an anti-stress effect. The anti-stress effect of this anti-stress agent can be confirmed, employing the rise in blood pressure, the rise in heart rate, and the change in Profile of Mood State (POMS), or the like as the indices, by determining the suppressing effects of the agent upon such rises and changes taken before and after the intake of the agent.

[0014] As the fermented sour milk contained as effective agent in the anti-stress agent of the present invention, lactic acid bacteria-fermented milk is used, of which great safety in its repeated daily use has conventionally been confirmed. The fermented sour milk may be prepared, for example, by first preparing a milk-containing stock solution.

[0015] The milk contained in the milk-containing stock solution may be of animal or vegetable origin. For example, animal milk such as cow's milk, goat's milk, sheep's milk, or horse's milk; or vegetable milk from soybeans or the like, may be used. The milk starting material may be whole fat or skim milk, whey, powdered milk, and/or reconstituted milk.

[0016] The milk-containing stock solution is not limited to a liquid wherein milk is dissolved or suspended and dispersed. The stock solution may be a solution-containing material such as paste prepared by mixing milk powders or a milk-containing material with water or a solution of salts. Additionally, a medium for lactic acid bacteria, yeast extracts, vitamins, minerals, sugars, lipids, flavors, or coloring agents may optionally be contained in the milk-containing stock solution.

[0017] Subsequently, the milk-containing stock solution is fermented with lactic acid bacteria, or symbiotically fermented with lactic acid bacteria and yeast for the purpose of improving flavor of the produced functional food, thereby obtaining the fermented sour milk.

[0018] The lactic acid bacteria are preferably lactic acid bacteria of the genus *Lactobacillus*. For example, *Lactobacillus helveticus, Lactobacillus delbruekii subsp. bulgaricus, Lactobacillus acidophilus, Lactobacillus fermentum*, or *Lactobacillus casei subsp. casei* may be used. Specifically, a strain such as *Lactobacillus helveticus* ATCC8205, *Lactobacillus helveticus* ATCC55796, *Lactobacillus delbruekii* subsp. *bulgaricus* ATCC11842, *Lactobacillus acidophilus* ATCC4356, *Lactobacillus fermentum* ATCC14931, or *Lactobacillus casei subsp. casei* ATCC393 may be used. Among these, *Lactobacillus helveticus* is particularly preferred.

[0019] The yeast which can be used for symbiotic fermentation with the lactic acid bacteria may be yeast of the genus *Saccharomyces*, genus *Candida*, or the genus *Kluyveromyces*. For example, a strain such as *Saccharomyces cerevisiae, Candida utilis*, or *Kluyveromyces marxianus var. lactis* may be used.

[0020] Culture conditions for fermentation include sterilizing the milk-containing stock solution under heating, cooling the sterilized stock solution to the predetermined culturing temperature, and admixing a starter consisting of previously cultured lactic acid bacteria, or of previously cultured lactic acid bacteria and the yeast, to the stock solution. The culturing temperature may be 20 to 50 °C, preferably 30 to 45 °C, and the culturing time may be 3 to 48 hours, preferably 6 to 24 hours. Termination of the culture can be determined with the number of lactic acid bacteria of 10^8 cells/g or more, and the acidity of the lactic acid of 1 or higher. The amount of the lactic acid bacteria starter to be inoculated for the culture is preferably 10^5 cells/g to 10^7 cells/g in terms of lactic acid bacteria with respect to the medium.

[0021] The anti-stress agent of the present invention may be in the form of the above cultured solution per se containing the fermented sour milk as an effective ingredient, or in the form of the cultured solution from which components other than fermented sour milk and lactic acid bacteria have suitably been separated. In either case, the lactic acid bacteria or the lactic acid bacteria together with the yeast therein are kept alive, i.e., in a live form. Alternatively, the cultured solution or the separated cultured solution may be sterilized under heating up to 80 °C to prepare an anti-stress agent in a sterilized form. Further, the above sterilized or non-sterilized cultured solution may be purified to obtain fermented sour milk; may be powdered by lyophilizing, spray drying, or drying in a drum dryer; or may further be formed into tablets using vehicles or carriers.

[0022] The anti-stress agent of the present invention may be administered orally to human or animals at any time such as before suffering stress, under stress, and after suffering stress, and may be administered even daily. The upper limit of the effective dose of the anti-stress agent is not particularly limited, and may suitably be selected. But the effective dose for sufficiently mitigating and preventing stress is, when the anti-stress agent is administered to human, preferably not less than 0.1 g per kilogram of body weight per day in terms of dried product of the fermented sour milk.

[0023] The functional food having the anti-stress effect of the present invention contains the above-mentioned anti-stress agent. Therefore, the anti-stress agent contained therein may be in the form of the cultured solution per se as mentioned above or after the suitable processing, with the lactic acid bacteria or the lactic acid bacteria together with the yeast being alive; in the sterilized form prepared by sterilizing under heating up to 80 °C; or in the powdered form prepared by lyophilizing, spray drying, or drying in a drum dryer.

[0024] The functional food of the present invention is not particularly limited as long as it contains the anti-stress agent as mentioned above. The anti-stress agent may be added after the food production, during the food

production, or at any stage of the food production. Further, the functional food of the present invention may suitably contain sugars, proteins, lipids, vitamins, minerals, flavors, or coloring agents in addition to the anti-stress agent, depending on the type of the food.

[0025] The amount of the anti-stress agent contained in the functional food of the present invention is not particularly limited, but is usually in the preferred range of 10 to 100 w/w% in terms of fermented sour milk.

[0026] The functional food of the present invention may be in the form of yogurt, milk-containing acidified beverages, cheese, processed foods containing fermented sour milk, healthy foods, or powdered or granulated foods.

[0027] The effective amount of the functional food of the present invention for mitigating and preventing stress is, in the case of human, preferably not less than 0.1 g per kilogram of body weight per day in terms of dried product of the fermented sour milk.

[0028] The anti-stress agent and the functional food of the present invention can be used for manufacture of a drug for the treatment of stress in the form of solid or liquid, specifically, in the form of tablets, granules, or nutrient supplementary drinks.

[0029] The anti-stress agent and the functional food having the anti-stress effect of the present invention contain as effective agent fermented sour milk prepared by fermentation with lactic acid bacteria. Accordingly, they are highly safe, can be ingested repeatedly and daily, and have effects of mitigating and preventing mental and physical symptoms caused by stress.

Examples

[0030] The present invention will now be explained in more detail with reference to Examples, but the present invention is not limited thereto.

Example 1

[0031] 2 kg of skim milk (solid content of 9 weight %) sterilized by heating up to 90 °C were inoculated with 40 g of a starter of previously cultured *Lactobacillus helveticus* ATCC8205, and cultured at 37 °C for 24 hours to prepare the secondary starter. Next, 4.5 kg of skim milk powders were dissolved in 45.5 kg of water, and the resulting solution was sterilized by heating up to 90 °C and cooled down to the room temperature. Then the solution was inoculated with the secondary starter, and cultured at 37 °C for 24 hours to obtain about 52 kg of fermented sour milk. After sterilizing the fermented sour milk at 80 °C for 10 minutes by heating, Aspartame (trade name, manufactured by Ajinomoto K.K.) was admixed to the fermented sour milk in an amount of 0.04 weight % of the total weight for facilitating drinking, thereby obtaining an anti-stress agent. As to the obtained anti-stress agent, the following experiments were conducted.

Experiment 1

[0032] The above mentioned anti-stress agent and a control consisting of a mixture of skim milk and lactic acid of the same concentration as the fermented sour milk, were given as a drink to fifteen (15) panels of healthy individuals (7 males, 8 females, age of 24 to 32), and mental arithmetic test as described below was conducted on the panels.

[0033] The panels were given one of the anti-stress agent and the control in an amount of 100 g each at 6:00 p.m. and 12:00 p.m. on the day before the measurement, 7:00 a.m. on the day of the measurement, and 30 minutes before the commencement of the test (10:00 a.m.), i.e. a total of four (4) times in a total amount of 400 g. Then on a different set of days, the panels were given the other of the anti-stress agent and the control according to the same timetable. The tests were conducted as a blind test so that the order of doses would not affect the test results. In order to adapt the panels to the testing environment, the panels entered the testing room ten minutes before the commencement of the test, and calmed down to resting conditions before the test was started. When the test was started, the panels were kept under the resting conditions for fifteen (15) minutes, and then placed under the calculation work for thirty (30) minutes from the fifteenth (15th) minute to the forty-fifth (45th) minute from the commencement of the test. During the testing time (from 0 minute to the 45th minute) , the blood pressure of the panels was measured continuously. Further, before the commencement of the test and after the termination of the calculation work, the panels were evaluated according to Profile of Mood State (POMS) method, which can evaluate the psychological conditions with the passage of time, in order to know their psychological conditions. The results of the tests were shown as follows: as to the blood pressure, by the change from the blood pressure in the resting condition; and as to the POMS, by the change from the points before the commencement of the test. The statistical differences were determined by paired t-test.

[0034] The fluctuation in the diastolic blood pressure during the testing period (from 0 minute to the 45th minute) is shown in Fig. 1. The diastolic blood pressure was elevated with the load of calculation work. In the case wherein the panels were given the anti-stress agent of the present invention, the rise in the diastolic blood pressure during loading of the calculation work stress was suppressed. Particularly, at the twentieth (20th) minute after the commencement of the test, the rise in the diastolic blood pressure was significantly suppressed as compared to that in the case of the controls with the significance level of 5 % (*).

[0035] The results of POMS test are shown in Fig. 2. It was recognized that, by comparing the results of the POMS test before the commencement of the test and after the calculation work, tension and depression

tended to be relaxed, fatigue tended to be mitigated, and emotional derangement tended to be stabilized, in the case wherein the panels were given the anti-stress agent of the present invention. In particular, tension was significantly relaxed as compared to the controls with the significance level of 5 % (*).

Experiment 2

[0036] Four (4) panels of healthy individuals (3 males, 1 female, age of 25 to 35) were given the anti-stress agent as a drink as mentioned above for seven (7) days in a row, and the mental arithmetic test was conducted before and after the uptake of the anti-stress agent.

[0037] On the first (1st) day, the mental arithmetic test was conducted on the panels from 10:30 a.m. in the same way as in Experiment 1. Subsequently, the panels were given 100 g of the anti-stress agent in the afternoon of the same day. From the second (2nd) day through the sixth (6th) day in a row, they were given 100 g of the anti-stress agent in the morning and in the afternoon. On the seventh (7th) day, the panels were given 100 g of the anti-stress agent at 7:00 a.m., and the same mental arithmetic test as in Experiment 1 was conducted on the panels from 10:30 a.m. The results of the test were shown by the change in the blood pressure and the heart rate from their resting conditions. The statistical differences were determined by paired t-test.

[0038] The fluctuation in the diastolic blood pressure during the testing period (from 0 minutes to the 45th minutes) is shown in Fig. 3. The diastolic blood pressure was elevated with the load of calculation work. In the test after the panels were given the anti-stress agent of the present invention for one week in a row, the rise in the diastolic blood pressure during loading of the calculation work was suppressed as compared to that in the test conducted before the panels were given the anti-stress agent. Further, the fluctuation of the heart rate during the testing period (from 0 minute to the 45th minute) is shown in Fig. 4. The heart rate was elevated with the load of the calculation work. In the test after the panels were given the anti-stress agent of the present invention for one week in a row, the rise in the heart rate during loading of the calculation work was suppressed, as compared to that in the test conducted before the panels were given the anti-stress agent.

Example 2

[0039] The fermented sour milk prepared in Example 1 was sterilized under heating at 80 °C for 10 minutes. After that, starting materials were mixed to obtain a mixture having the composition of 75 weight % of the sterilized fermented sour milk, 13.3 weight % of 3 wt% HM pectin solution, 3.03 weight % of 30 wt% sodium citrate solution, 4.5 weight % of 1 wt% Aspartame solution, 0.25 weight % of blended flavor, and 3.92 weight % of water. The mixture was sterilized by heating up to 85 °C, and then charged in brown bottles by 100 g each while hot, thereby producing fermented sour milk beverage. The obtained fermented sour milk beverage was subjected to the tests as in Example 1 to reveal that it has similar effects on blood pressure, heart rate, and POMS.

Claims

1. An anti-stress agent comprising as effective agent at least one fermented sour milk.

2. The anti-stress agent of claim 1 wherein said fermented sour milk includes fermented sour milk prepared by fermenting an animal or vegetable milk starting material with lactic acid bacteria of the genus *Lactobacillus*.

3. The anti-stress agent of claim 1 wherein said lactic acid bacteria of the genus *Lactobacillus* are *Lactobacillus helveticus*.

4. The anti-stress agent of claim 3 wherein said *Lactobacillus helveticus* is of a strain deposited with the accession number ATCC8205 or ATCC55796.

5. The anti-stress agent of any one of claims 1 to 4 wherein said fermented sour milk includes fermented sour milk prepared by fermenting an animal or vegetable milk starting material with both lactic acid bacteria of the genus *Lactobacillus* and a yeast.

6. The anti-stress agent of any one of claims 1 to 5 wherein said fermented sour milk is in a live form.

7. The anti-stress agent of any one of claims 1 to 5 wherein said fermented sour milk is in a sterilized form.

8. A functional food having anti-stress effect comprising the anti-stress agent of any one of claims 1 to 7.

9. The functional food of claim 8 wherein content of said anti-stress agent is 10 to 100 W/W% in terms of the fermented sour milk.

10. The functional food of claim 8 or 9 wherein said functional food is in the form selected from the group consisting of yogurt, milk-containing acidified beverages, cheese, processed foods containing fermented sour milk, healthy foods, powdered foods, and granulated foods.

11. The functional food of any one of claims 8 to 10 further comprising a component selected from the group consisting of sugars, proteins, lipids, vita-

mins, minerals, flavors, coloring agents, and mixtures thereof.

12. Use of the anti-stress agent of any one of claims 1 to 7 or of the functional food of any one of claims 8 to 11 for the manufacture of a drug for the treatment of stress.

13. The use of claim 12 wherein said anti-stress agent or the functional food is orally administered at a dosage of not less than 0.1 g per kilogram of body weight per day in terms of dried product of the fermented sour milk.

Fig. 1

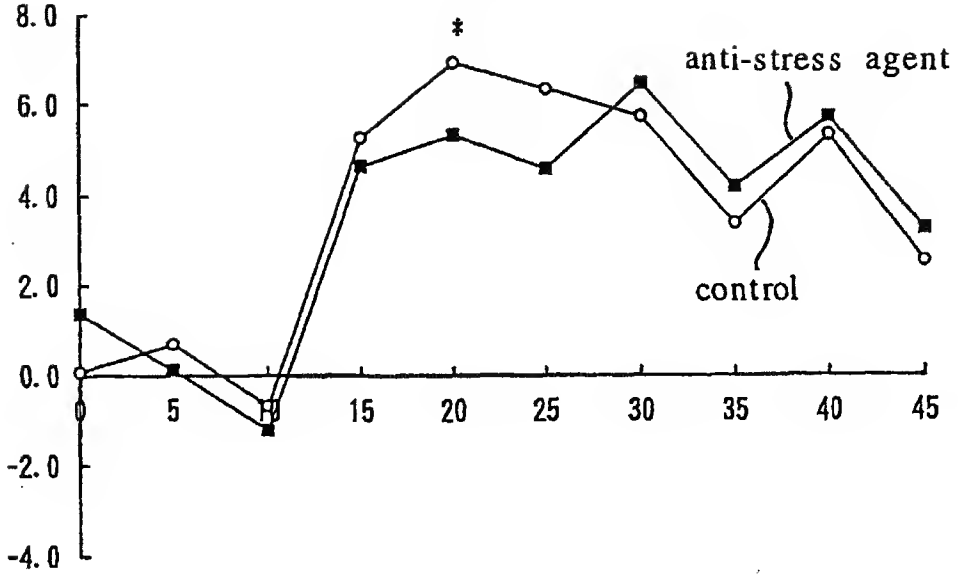


Fig. 2

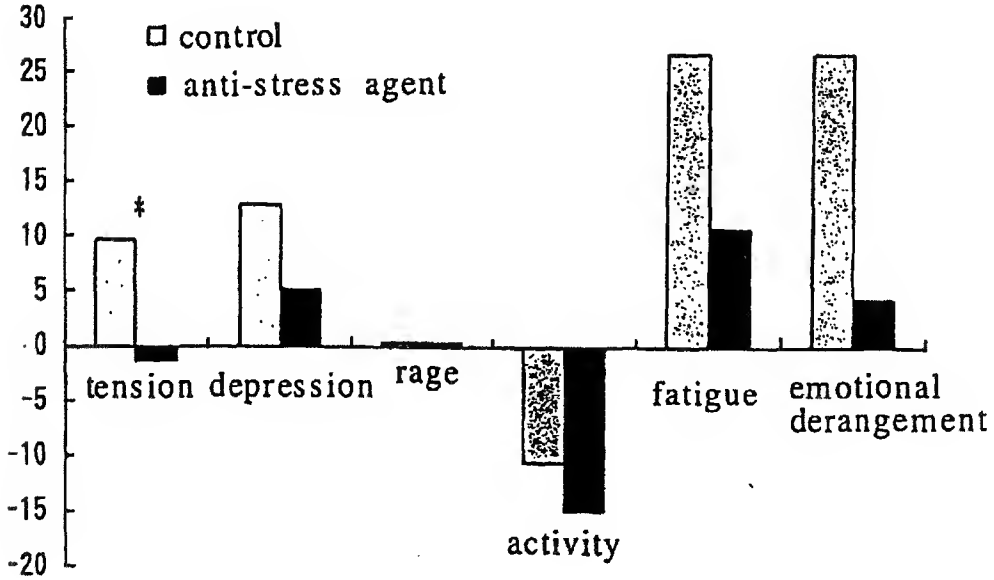


Fig.3

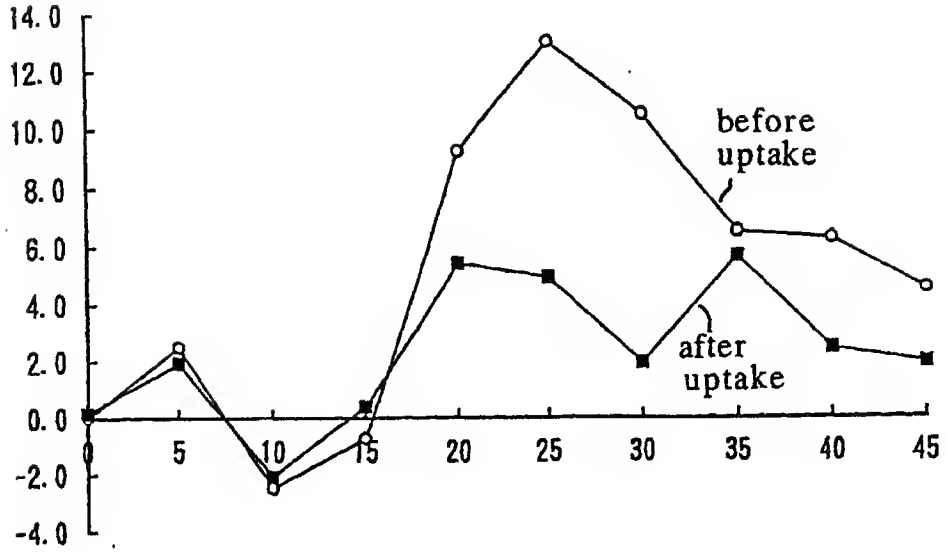
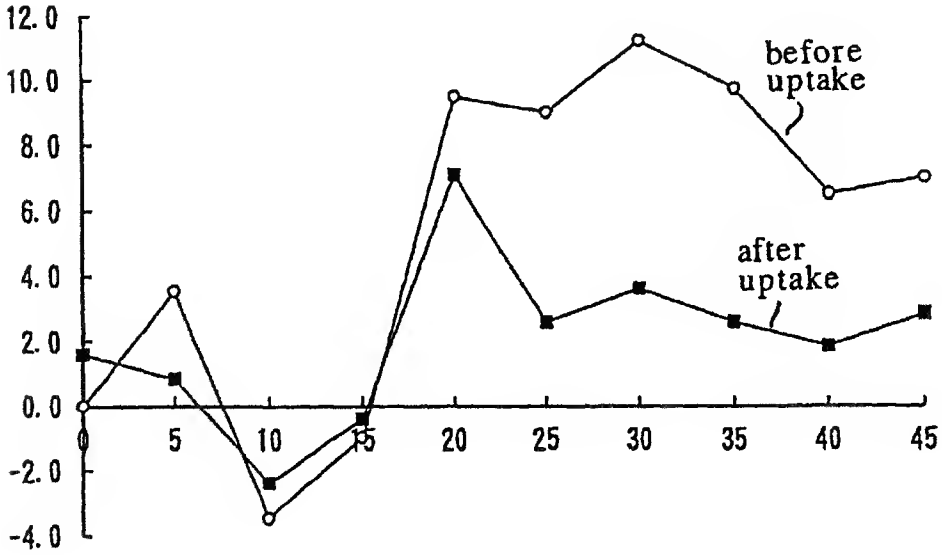


Fig.4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP97/02728

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl^6 A61K35/20, A23L1/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int. Cl^6 A61K35/00-35/84, A23L1/00-1/48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1940 - 1997
Kokai Jitsuyo Shinan Koho 1971 - 1997

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA(STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP, 7-123977, A (The Calpis Food Industry Co., Ltd.), May 16, 1995 (16. 05. 95), Par. No. 2 & EP, 652285, A & US, 5541111, A	1-3, 5-13
A	JP, 61-53216, A (The Calpis Food Industry Co., Ltd.), March 17, 1986 (17. 03. 86), Claim; page 2, upper right column, lines 2 to 10, lower left column, line 14 to lower right column, line 2 (Family: none)	1 - 13
A	JP, 4-5236, A (Yakult Honsha Co., Ltd.), January 9, 1992 (09. 01. 92), Claim; page 2, lower left column, lines 17 to 19 (Family: none)	1 - 13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
October 22, 1997 (22. 10. 97)	November 5, 1997 (05. 11. 97)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

JP1198978

Title:
OVERHEAD PARKING AREA DEVICE

Abstract:

PURPOSE:To sharply reduce a parking cost, by a method wherein suspension beam materials and rails are spanned in the upper space zone of a lower stage parking area, and elevating and running pallets, a running truck, vertical and horizontal drive means are mounted to form upper and middle stage parking areas. CONSTITUTION:Suspension beam materials 5... and rails 6 and 6 are spanned to stays 1..., erected in lower stage parting areas C and C, with a vertical distance therebetween. Elevating pallets 10... having vertical drive means D... are hung down from the suspension beam materials 5... to form upper stage parting areas A.... A running truck 20 having a horizontal drive means E is engaged with the rails 6 and 6, and running pallets 23 and 23 having the vertical driven means D... hung down from the rails to form middle stage parking areas B and B.

EP1302207

Title:
Method for preparing an antihypertensive agent containing a dipeptide as active ingredient

Abstract:
A method for producing an antihypertensive agent containing an effective amount of dipeptide Tyr-Pro and/or a pharmaceutically acceptable salt thereof, the effective amount being from 0.05 to 10mg/kg body weight/day.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 302 207 A2

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
16.04.2003 Bulletin 2003/16

(51) Int Cl.[7]: **A61K 38/05**, C12P 21/06
// (C12P21/06, C12R1:225)

(21) Application number: **02080463.9**

(22) Date of filing: **01.08.1997**

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **02.08.1996 JP 20466596**

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
97305820.9 / 0 821 968

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Remarks:
This application was filed on 18 - 12 - 2002 as a divisional application to the application mentioned under INID code 62.

(54) Method for preparing an antihypertensive agent containing a dipeptide as active ingredient

(57) An antihypertensive agent containing an effective amount of dipeptide Tyr-Pro and/or a pharmaceutically acceptable salt thereof, the effective amount being from 0.05 to 10mg/k body weight/day, and a method for producing the same.

EP 1 302 207 A2

Description

[0001] The present invention relates to an antihypertensive agent containing as an active ingredient a dipeptide having antihypertensive effect, which agent may be utilized for medicine, foods for specified health use and healthy foods, and which agent exhibit the effect even in a small dose. The present invention also relates to a method for preparing such antihypertensive agent.

[0002] Angiotensin converting enzyme (ACE), having close relationship with manifestation of hypertension, exists mainly in lungs or angioendothelial cells. ACE is known to exhibit a strong hypertensive effect by cleaving angiotensin I produced by renin, to produce angiotensin II, a bioactive peptide which causes contraction of a smooth muscle of a blood vessel. In addition, ACE degrades and inactivates bradykinin which has antihypertensive effect. Accordingly, ACE has a hypertensive effect, and it is thus believed that blood pressure may be suppressed by inhibiting the activity of this enzyme.

[0003] There have been found substances having ACE inhibiting ability among various natural products or synthetic products. Some of such substances have already been put to practical use as an antihypertensive agent. For example, captopril (D-2-methyl-3-mercaptopropanoyl-L-proline) is a well-known synthetic chemical product having ACE inhibiting ability. However, special attention must be paid at all times to safety aspects of these synthetic chemical products.

[0004] As natural products, it has been reported that various anti ACE peptides are contained in milk protein, soybean protein or fish meat protein. These natural ACE inhibitory substances are proposed for practical use as antihypertensive agents having low toxicity and great safety. However, most of these antihypertensive peptide are contained only in small amount in such natural products and therefore sufficient effect cannot be expected in practical oral intake. In addition, most of the peptides do not have strong antihypertensive effect even if the peptides have strong ACE inhibition activity.

[0005] Recently, a report has been made on two tripeptides having strong ACE inhibiting activity, Val-Pro-Pro and Ile-Pro-Pro, derived from lactic acid bacteria-fermented milk (J. Dairy Sci. 78:777-783). Further, strong antihypertensive effect of these tripeptides has been confirmed in spontaneously hypertensive rats (SHR) (J. Dairy Sci. 78:1253-1257). However, since the tripeptides is produced by proteinase which is produced by lactic acid bacteria as lactic acid fermentation proceeds in milk, the resulting amount of tripeptides tends to vary depending on the conditions of fermentation. It is thus difficult to obtain the tripeptides in a stable amount.

[0006] It has also been reported that yogurt produced by lactic acid bacteria fermentation of milk as a raw material has antihypertensive effect (Japanese Laid-open Patent Application No.95-123977). However, the active substance for antihypertensive effect has yet been unknown.

[0007] Therefore, it is desired to provide a natural antihypertensive peptide, which is specified as an active substance, can be produced in an industrially stable manner, is effective in a small dose, and has greater safety.

[0008] It is an object of the present invention to provide an antihypertensive agent to be used for medicines, foods for specified health use and healthy foods, which is highly safe and is effective in oral dosage, and a method for producing the same.

[0009] It is another object of the present invention to provide an antihypertensive agent which is effective even in a low oral dosage, and a method for producing such agent by purifying the above-mentioned antihypertensive agent.

[0010] According to the present invention, there is provided an antihypertensive agent comprising an effective amount of peptides selected from the group consisting of a dipeptide Tyr-Pro, a pharmaceutically acceptable salt of the dipeptide Tyr-Pro, and mixtures thereof, said effective amount being from 0.05 to 10mg/kg body weight/day.

[0011] According to the present invention, there is also provided a method for preparing the antihypertensive agent comprising the step of culturing lactic acid bacteria of the genus *Lactobacillus* with a medium containing a peptide and/or protein including an amino acid sequence Tyr-Pro, to obtain a cultured liquid containing dipeptide Tyr-Pro.

[0012] The antihypertensive agent of the present invention contains an effective amount of dipeptide Tyr-Pro and/or a salt thereof. The salt may be enumerated by a pharmaceutically acceptable salt, including an inorganic acid salt, such as hydrochlorate, sulfate or phosphate, and an organic acid salt, such as citrate, maleate, fumarate, tartarate or lactate.

[0013] The antihypertensive agent of the present invention containing the dipeptide Tyr-Pro and/or the salt thereof as an active ingredient may be produced by a method of treating a food material containing a peptide and/or a protein including the amino acid sequence Tyr-Pro with a proteinase, or a publicly known organic synthesis. More preferably, the antihypertensive agent may be produced by culturing lactic acid bacteria, preferably lactic acid bacteria of the genus *Lactobacillus,* in a medium containing a peptide and/or a protein including the amino acid sequence Tyr-Pro, thereby obtaining a cultured liquid containing the dipeptide Tyr-Pro. That is, the dipeptide Tyr-Pro may be obtained by cultivation treatment of the medium with lactic acid bacteria, preferably lactic acid bacteria of the genus *Lactobacillus.*

[0014] The lactic acid bacteria of the genus *Lactobacillus* may include, for example, *Lactobacillus helveticus, Lactobacillus delbruekii* subsp. *Bulgaricus,* and *Lactobacillus acidophilus.* Of these, *Lactobacillus helveticus* is particularly preferred.

[0015] The medium is not particularly limited provided that the medium contains a peptide and/or a protein including amino acid sequence Tyr-Pro, and may include mediums derived from various food materials containing animal milk proteins such as animal whole milk, skim milk, and casein of an animal milk, and food materials containing vegetable proteins such as corn, corn protein, wheat, wheat protein, soybean, delipidized soybean and soybean protein; and commercially available mediums for lactic acid bacteria such as Briggs liver broth and MRS broth. Further, the medium may also be an aqueous solution containing natural food materials containing animal milk proteins and/or vegetable proteins including amino acid sequence Tyr-Pro, to which other mediums for lactic acid bacteria, a yeast extract, vitamins or minerals have optionally been added.

[0016] The culturing of the lactic acid bacteria may be performed by adding pre-cultured lactic acid bacteria starter to the medium which have been previously heat-sterilized and cooled to the predetermined temperature for incubation. The inoculation amount of the lactic acid bacteria starter may preferably be 10^5 to 10^7 cells of lactic acid bacteria/ml medium. The temperature for incubation is usually 20 to 50°C and preferably 30 to 45°C. The incubation time is usually 3 to 48 hours and preferably 6 to 24 hours. Particularly, it is preferred to perform cultivation in the medium having pH in a range of 3.5 to 7, more preferably 4 to 5, in order to perform cultivation of lactic acid bacteria efficiently. Further, it is preferred to perform pH-stat cultivation maintaining pH in a range of 4 to 7. The incubation may be terminated, without restriction, when the number of lactic acid bacteria exceeds 10^8 cells/ml.

[0017] Further, the cultured liquid containing dipeptide Tyr-Pro obtained by the aforementioned method may be centrifuged, and the resulting supernatant may be subjected to purifying treatment with a reverse-phase resin, for obtaining an antihypertensive agent in which the content of the active ingredient, dipeptide Tyr-Pro, is increased.

[0018] The centrifugation may preferably be performed, for example, at 5,000 to 20,000rpm for 1 to 10 minutes. The centrifugation may also be performed in a centrifugator.

[0019] The purifying treatment with a reverse-phase resin may be performed by absorption and elution of the dipeptide with a reverse-phase resin, and/or by reverse-phase chromatography, thereby increasing purity of dipeptide Tyr-Pro. The absorption and elution with the reverse-phase resin may be performed by, for example, treating the cultured liquid by a column method or a batch method with a resin such as "Preparative C18" (manufactured by WATERS INC.) as a reverse-phase resin, and then eluting the absorbed fraction with a polar solvent such as water, methanol, ethanol, 1-propanol, 2-propanol or acetonitrile, preferably an aqueous solution containing 20 v/v% of acetonitrile, followed by evaporation of the solvent, for concentrating dipeptide Tyr-Pro.

[0020] The treatment by reverse-phase chromatography may be performed, without limitation, by publicly known reverse-phase high performance liquid chromatography (HPLC). HPLC may be performed by a linear gradient method using similar solvents to those enumerated in the absorption and elution with the reverse-phase resin, for obtaining dipeptide Tyr-Pro with high purity. The treatment by reverse-phase chromatography may be repeated plural times for further increasing the purity of the dipeptide Tyr-Pro.

[0021] The antihypertensive agent obtained by the method of the present invention is usually a mixture of peptides, and may contain other peptides than dipeptide Tyr-Pro. For use as foods and drinks, the cultured liquid containing the dipeptide Tyr-Pro and/or purified products thereof may be used directly. Alternatively, the agent may be powdered by freeze drying, spray drying or drum dryer drying, before use.

[0022] The effective amount of the antihypertensive agent of the present invention varies depending upon the age and condition of a patient, and is in a range of 0.05 to 10mg/kg body weight/day. It is preferable to administer 0.3 to 3.0mg/kg body weight/day. If the dose is not less than 0.05mg/kg body weight/day, sufficient effect may be expected. If the dose is not more than 10mg/kg body weight/day, the effect may be exhibited efficiently.

[0023] The dipeptide Tyr-Pro was orally administered to WKY rats showing normal blood pressure (18 weeks old, body weight of 320g, n=5) in an amount of 10mg/kg body weight/day continuously for one month, and no abnormality was found in behavior, appearance, blood pressure and autopsy of the rats. Foods fermented with *Lactobacillus* bacteria have been eaten by human beings for a long time, and it is therefore assumed that Tyr-Pro contained in the antihypertensive agent of the present invention has no problem in safety.

[0024] Since the antihypertensive agent of the present invention contains dipeptide Tyr-Pro as an active ingredient, it can reduce blood pressure in significantly low dose, compared with antihypertensive peptides derived from food materials reported to date. In addition, the antihypertensive agent of the present invention is highly safe, and thus can be used as medicine, foods for specified health use and healthy foods.

[0025] Since the method of the present invention includes culturing of lactic acid bacteria in a medium containing a peptide and/or a protein, the antihypertensive agent containing as an active ingredient dipeptide Tyr-Pro having great safety may be produced easily at low cost from a material such as natural peptides and proteins.

[0026] The present invention will be explained in further detail with reference to Examples which are given only for illustration and are not intended for limiting the invention.

Preparation Example 1

[0027] 10 kg of a 9 wt% aqueous solution of skim milk powders were inoculated with 300 g of fermented milk fermented with *Lactobacillus helveticus* CPN4 (FERM BP-4835) as a lactic acid bacteria starter. The inoculated solution was incubated at 37°C for 8 hours. When curd was formed and the pH reached 4.2, the incubation was terminated and the solution was cooled. In the obtained fermented milk solution, 1.0mg/100g of dipeptide Tyr-Pro was contained.

[0028] 10kg of the obtained fermented milk solution were centrifuged at 10,000rpm for 10 minutes, to retrieve 8.4 kg of whey. In the whey, 1.1mg/100g of dipeptide Tyr-Pro were contained.

[0029] *L. helveticus* CPN4 strain belongs to *L. helveticus,* and was deposited at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology on October 17, 1994, and has been accorded accession number FERM BP-4835. FERM BP-4835 has been accepted for deposit under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. All restrictions on the availability to the public of FERM BP-4835 will be irrevocably removed upon the granting of a patent.

Example 1

[0030] 120mg of the whey obtained in Preparation Example 1 were subjected to an absorption treatment through 10 sep-pak cartridges (WATERS INC.), and subsequently to an elution treatment under various conditions shown in Table 1 with an acetonitrile containing 0.1wt% of TFA (trifluoroacetic acid) (solution B) diluted with an aqueous solution containing 0.1wt% of TFA (solution A). A sample eluted without absorption and samples eluted under each condition were defined as Fractions 1 to 6, respectively. Each fraction was freeze-dried and dissolved in 40ml of physiological saline to prepare Samples 1 to 6.

[0031] The obtained whey before fractionation and Samples 1 to 6 were administered to spontaneously hypertensive rats (SHR: from CHARLES RIVER JAPAN INC., five rats per group, 20 to 22 weeks old, male) with a stomach sonde in an amount of 1ml/animal. The change in the maximal blood pressure after six hours was compared with that of a control group to which physiological saline was administered. The measurement of blood pressure was performed by tail cuff method using a noninvasive blood pressure measurement apparatus (trade name "PE 300", NARKO BIO-SYSTEMS, CO.,LTD.). As a result, a significant antihypertensive effect was observed in Sample 2 as shown in Table 1. Therefore, Fraction 2 was found to be active.

Table 1

Sample	Ratio of Solution B (%)	Blood Pressure Reduction After 6 hours (ΔSBP±SE)
Before Fractionation	-	-29.6±9.5 ***
Fraction 1	not absorbed	-9.6±5.7 *
Fraction 2	0-10	-22.3±3.2 *** (Active Fraction)
Fraction 3	10-15	-8.1±2.6
Fraction 4	15-20	1.0±6.3
Fraction 5	20-30	-8.8±8.5 *
Fraction 6	30-50	-7.6±5.3
Control (physiological saline)		1.9±8.1

***;P<0.001, *;P<0.05

[0032] Subsequently, Sample 2 was subjected to an elution treatment by reverse-phase high performance liqud chromatography (HPLC) under the following conditions according to the elution time shown in Table 2, to further fractionate Sample 2 into four Fractions 2-1 to 2-4. Application amount of Sample 2 in one elution was 1ml and elution was repeated 15 times . That is, a total of 15ml of Sample 2 was fractionated.

Pump, L6200 INTELLIGENT pump manufactured by HITACHI LTD.;
Detector, L4000 UV detector manufactured by HITACHI LTD.;
Column, WATERS MICROBONDASPHERE 5μC18 manufactured by NIHON MILLIPORE LTD., Tokyo, Japan;
Eluent, Solution A (0.1 wt% aqueous solution of trifluoroacetic acid (TFA)); and Solution B (acetonitrile containing 0.1 wt% of TFA);
Gradient, linear gradient from Solution B 0% (Solution A 100%) to Solution B 40% (Solution A 60%) (0 to 60 minutes) Flow rate, 1ml/min.

[0033] Fractions 2-1 to 2-4 were freeze-dried, and dissolved in 15ml of physiological saline to prepare Samples 2-1 to 2-4. These samples were orally administered to SHR with a stomach sonde in an amount of 1ml/animal, and the change in the maximal blood pressure after six hours was compared with that of a control group to which physiological saline was administered, in the same manner as the above, in order to examine antihypertensive effect. As a result, a-significant antihypertensive effect was observed in Fraction 2-2 as shown in Table 2. Therefore, Fraction 2-2 was found to be an active fraction.

Table 2

Sample	Elution Time (min)	Blood Pressure Reduction After 6 hours (ΔSBP±SE)
Fraction 2-1	10.5-16	-13.0±2.8 **
Fraction 2-2	16-19	-35.0±9.7 *** (Active Fraction)
Fraction 2-3	19-23	-2.8 ±5.3
Fraction 2-4	23-29	-3.6±5.6
Control (physiological saline)		2.6±6.0

***;P<0.001, **;P<0.01

[0034] 1ml of Sample 2-2 was subjected to elution treatment by the HPLC under the same conditions as those of the above except following the under-mentioned conditions:

Gradient, linear gradient from Solution B 5% (Solution A 95%) to Solution B 20% (Solution A 80%) (0 to 60 minutes)

[0035] This elution was repeated ten times, that is, 10ml in total of Sample 2-2 was subjected to the elution.

[0036] Each of the obtained Fractions 2-2-1 to 2-2-5 was freeze-dried and dissolved in 10ml of physiological saline to prepare Samples 2-2-1 to 2-2-5. These samples were orally administered to SHR with a stomach sonde in an amount of 1ml/animal, and the change in the maximal blood pressure after six hours was compared with that of a control group to which physiological saline was administered, in the same manner as the above, in order to examine antihypertensive effect. As a result, a significant antihypertensive effect was observed in Fraction 2-2-2 as shown in Table 3.

Table 3

Sample	Elution Time (min)	Blood Pressure Reduction After 6 hours (ΔSBP±SE)
Fraction 2-2-1	21.8	-6.6±5.5
Fraction 2-2-2	22.7	-23.0±6.2 *** (Active Fraction)
Fraction 2-2-3	24.0	-9.0±6.5 *
Fraction 2-2-4	25.2	-6.2±6.2
Fraction 2-2-5	25.8	-3.5±4.8
Control (physiological saline)		2.6±6.0

***;P<0.001, *;P<0.05

[0037] 1ml of Sample 2-2-2 was subjected to the elution treatment by the HPLC under the same conditions as those for treating Sample 2-2 for purifying the sample, and the amino acid sequence thereof from N-terminus was analyzed with "Automatic Protein Sequencer PPSQ-10 SYSTEM" manufactured by SHIMADZU CO. As a result, it was confirmed that amino acid sequence Tyr-Pro was contained. Alternatively, the purified peptide was hydrolyzed with 6N hydrochloric acid at the temperature of 105°C for 24 hours, and amino acid analysis was performed with high-speed amino acid analysis system (trade name "Amino Acid Analysis System" manufactured by NIHON BUNKOU KOUGYO Co.). As a result, it was confirmed that amino acids Tyr and Pro were contained in equal moles. It was therefore concluded that the obtained peptide was a dipeptide having the sequence Tyr-Pro.

Preparation Example 2

[0038] 100kg of a 9 wt% aqueous solution of skim milk powders were inoculated with 3kg of fermented milk fermented